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THE NATURE OF THE ACTIVITIES OF THE HUMAN SWEAT GLANDS[1]

EDWARD F. ADOLPH

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Received for publication July 30, 1923

1. The composition of human sweat has been the topic of numerous papers in the physiological and biochemical literature (1). The value of the sweat glands as excretory organs has been frequently investigated (2). It is now in order to describe how the sweat glands act as chemical mechanisms. The questions to be considered, therefore, are: *a*. What are the controlling factors in the composition of the sweat? *b*. What elaboration of solutions do sweat glands perform?

It is already known that the sweat is more dilute than the blood in respect to most dissolved substances (1), (2). This property indicates its appropriate nature for cooling the body by evaporation. It is also known that there is some variation in the dilution of sweat from time to time (3), and it has ordinarily been supposed that this dilution is correlated with the activities of the body, such as amount of work performed during the sweating (4). That there is also a reciprocal activity between the sweat glands and the kidneys is readily recognized (5). The only other glands which have been studied from these points of view are the salivary glands and the milk glands.

2. The methods used in this study were as follows: Sweat was collected from six different subjects, during exposure to high temperatures in the psychrometric room located at the Pittsburgh Experiment Station of the U. S. Bureau of Mines. The conditions of temperature and humidity were constant within 1°F. and 1 per cent throughout each test. In no cases was work of any kind performed; the subjects sat upright; no clothing was worn above the waist. In several cases before sweat was collected the area of skin was thoroughly cleansed, but contrary to the experience of Hunt (6), little difference was noted in the

[1] Published by permission of the Director, U. S. Bureau of Mines.

composition of the sweat whether this cleansing was performed or not. Sweat was collected by lightly passing a beaker over the surface of the skin, and as soon as the collection was completed the contents of the beaker, which usually amounted to 5 or 10 cc., were placed in a test tube and tightly stoppered. Analyses were made the same day. The hydrogen ion concentration was determined by diluting the sweat 20 times with neutralized distilled water, and comparing the samples with the phosphate standards of Sörensen (7), using phenol red as indicator. Specimens too acid to give a comparable color with this indicator were not studied further. In the acidity data given, no correction has been made for dilution or temperature. The escape of carbon dioxide from the specimens was not prevented, as this escape was entirely accomplished before the samples had been placed in test tubes. The samples were collected at various times during the course of exposure to high temperatures, and these times were accurately noted. Chloride concentrations in the sweat were determined by titrating 5 cc. or less of the sweat by the Harvey (8) modification of Volhard's method. Hemoglobin concentrations were measured by the spectrophotometric method (9). The rate of sweating was estimated by weighing the subjects to an accuracy of 10 grams just before and after each test.

3. Most of the samples were collected by Dr. W. J. McConnell, Dr. F. Flinn and Mr. W. B. Fulton, while carrying on other tests. I desire to thank them for their coöperation. In addition, Doctor McConnell has been very attentive in arranging the tests, and Mr. Fulton has carried out some of the chloride titrations. I am indebted to Mr. W. P. Yant for the hemoglobin determinations. Dr. R. R. Sayers, Dr. McConnell and Mr. F. C. Houghten have kindly placed at my disposal the extensive correlative data collected in numerous tests.

VARIATIONS IN THE COMPOSITION OF SWEAT. 1. It was first desired to find whether the composition of the sweat varied with the time during which sweat was secreted. Such data have been plotted in figure 1. It will be seen that the chloride concentration increased markedly up to a certain maximum, and then remained constant. In other words, the initial sweat contains very little dissolved substance. This maximum is approximately the same for all subjects and is about 0.08 M. That the sweat is more dilute at first indicates that relatively little error is introduced by the presence of residual chlorides in the skin. Kittsteiner (3) has suggested that the chloride concentration in sweat depends on the rate of secretion, and in these experiments the rate of sweating increased from the beginning to the end of the test.

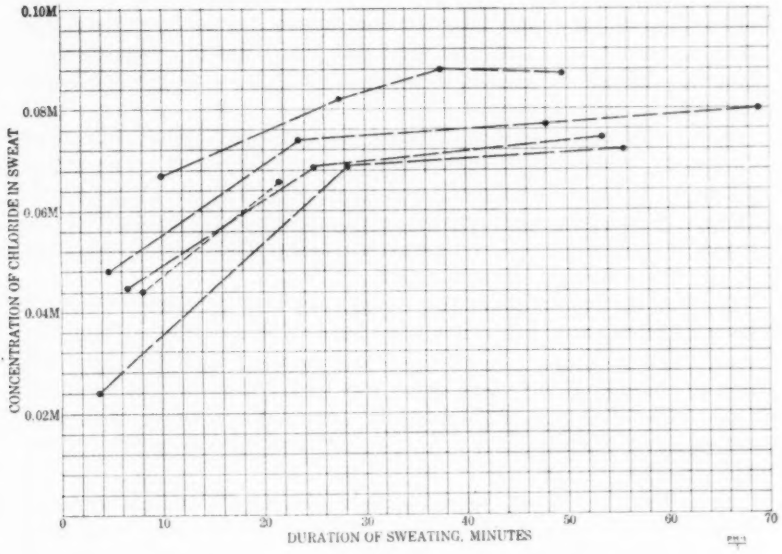

Fig. 1. Variation in concentration of chloride in sweat during 5 experiments on subject W. B. F. Atmospheric conditions, 105°F., saturated.

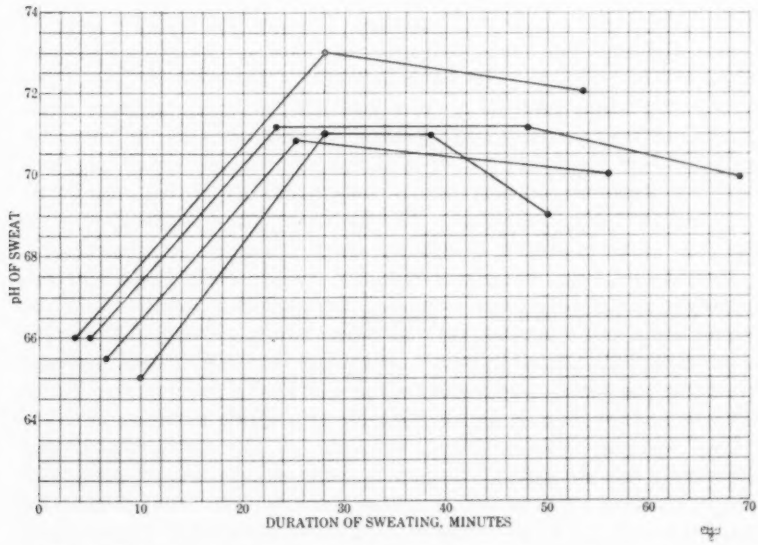

Fig. 2. Variation in the reaction of sweat during 4 experiments on subject W. B. F. Conditions, 105°F., saturated.

The increase in concentration during sweating has been confirmed upon all 6 subjects, and the maximum attained after 30 minutes has always been between 0.065 M and 0.085 M. Hunt (6), who obtained constant chloride contents of 0.020 M to 0.025 M, must have collected only the earliest samples.

The hydrogen ion concentration of the sweat was found to decrease during the course of the sweating, as shown in figure 2. The higher alkalinity produced toward the end never exceeded that of the blood. Sweat, as has long been known, is characteristically acid. The increase in alkalinity seems to be correlated with the increased alkalinity of the blood during the same period.

2. Does the composition of the sweat vary with the area of the body from which the sweat is collected? Samples were collected from the chest, arms and face. In each case it was found that the concentration of chloride in the chest sweat was higher than in that from the arms, while that from the face tended to be the same as that from the chest. Similar results were obtained by Kittsteiner (3) and by Talbert (4).

In contrast to this, the secretions from the chest and arms were less alkaline than those from the face, although all of them progressed simultaneously toward a more alkaline fluid. Talbert (10) also noted such a regional variation in reaction.

3. It was also desired to compare the composition of the sweat produced under varying atmospheric conditions. As was to be expected, the sweat as collected was more highly concentrated in proportion to the humidity which prevailed; the data upon this phase of the problem have already been published (11). The relative humidities tested were 30 per cent, 60 per cent and 100 per cent. It seems probable, therefore, that the concentration of substances in the sweat as elaborated by the sweat glands is the same under all conditions, and that the apparent concentration in low humidities is due entirely to evaporation during collection. The hydrogen ion concentration does not vary with the relative humidity.

Considering only the results obtained under conditions where the atmosphere was completely saturated with moisture, there is no apparent change in concentration with temperature, although Talbert (4) believes the skin temperature is a controlling factor in the ash of sweat. From the present data one would conclude that at a definite time during the course of sweating the sweat glands are elaborating a uniform product.

THE NATURE OF THE SWEAT GLANDS. 1. The sweat glands are apparently set into activity through nervous reflexes, which are usually of a generalized character (1). All areas of the body sweat practically simultaneously, in spite of differential exposure, although under special circumstances local sweating has been recorded.

But there is apparently no limit to the rate at which the sweat glands can work. As shown in figure 3, the amount of sweat produced is a function of the external conditions, and as a measure of these conditions the "effective temperature" has been used, the validity of which has been demonstrated by Houghten and Yagloglou (12), (13). Whether the increased sweat is produced by the excitation of more glands or by the increase in activity of the same glands, is unknown. The stimulus to increased activity is also unknown although the rate of activity, I find, is closely correlated with the superficial body temperature.

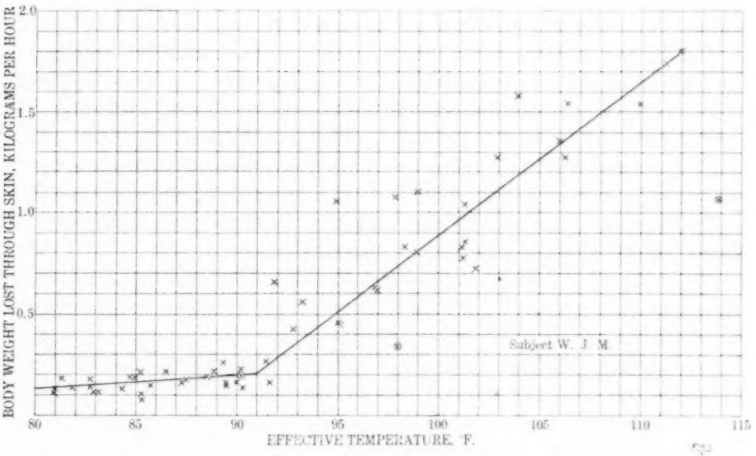

Fig. 3. Variation in the body weight loss per hour plotted against the effective temperature. In most tests the atmosphere was saturated, when the effective temperature, wet bulb temperature, and dry bulb temperature are the same. Subject W. J. M.

2. The sweat glands are concerned with the excretion of a fairly uniform, dilute fluid. The total concentration of sweat is less than the total concentration of blood. The chloride concentration is less than that of blood, and the acidity is always greater than that of the blood. Nevertheless, the chloride and acidity are probably influenced by the composition of the blood, for the sweat is more concentrated in chloride and in alkali after the blood has become concentrated and more alkaline.

In order to arrive at some conception of what the sweat glands do, we may compare them with the kidneys. In both cases an acid product is usually elaborated. In the kidneys, however, concentrated

solutions are produced except during water diuresis (5). The kidneys are concerned with the conservation of the body's water; the sweat glands are concerned with the manufacture of a dilute fluid which shall easily evaporate. The sweat glands are conservers, therefore, of the body's supply of salts and other dissolved substances.

As long as sweating continues, a high concentration of chlorides is present in both sweat and urine. The urinary volume diminishes, however, during the sweating (5). After sweating has ceased the urinary chloride is greatly diminished, with a marked oliguria. The reciprocal relation of sweat glands and kidneys is due to the fact that the kidneys are relieved from excreting the chloride and water that the sweat glands have eliminated.

3. The variation in the kidney's product is known to be very great, so that the kidneys are capable of excreting water in large amounts. The sweat glands, on the contrary, are not capable of excreting dissolved substances in concentrated solutions. Talbert (4) has shown that there is a variation in the ash content of sweat with various types of work. This variation, however, is not large, and may possibly be partly accounted for by the variation in the excretion at various times from the same sweat glands, as noted above.

One important variable in the sweat is the hydrogen ion concentration, and this seems to be correlated with the changes in the blood's composition which takes place simultaneously (14). Similar changes have been demonstrated by Starr (15) for the saliva, and they are exactly parallel to the alveolar CO_2 concentration.

The ash content, too, may be considered to be correlated with the blood's composition, for the hemoglobin concentration has been found in these experiments to increase during sweating; and probably the blood's chloride concentration increases proportionally. Similar correlations have been found by Wilbrand (16) and Bogendörfer (17). In one experiment 500 ccc. of water were drunk. No dilution of the sweat resulted, but the concentration of chloride increased at its usual rate.

4. The sweat glands, as all glands, are organs which perform a certain amount of work in separating water from other substances. The amount of separation which they actually accomplished is comparatively small. The work may be calculated by the method of Dreser (18) and Rhorer (19) by comparing the concentrations of dissolved substances in blood and secreta. It has been found by Plaggemeyer and Marshall (2) that urea and ammonia, as well as water, are more concentrated in the sweat than in the blood. All other things, as far as is known, are slightly diluted in the sweat. The presence of high concentrations of urea and ammonia in the sweat is probably

correlated with their universal permeability. They go wherever water goes.

5. The dilution of sweat is not nearly as great as, for instance, in the case of the urine during water diuresis. Moreover, it seems to be impossible to produce significant variations in the sweat's composition, by experimenting upon the normal organism. Although, therefore, the blood's composition influences the product of the sweat glands to a small extent, these glands are not under the influence of the blood's concentrations in the way that the kidneys are.

SUMMARY

1. The composition of sweat from the human body varies with the region and with the duration of sweating. It does not vary with the atmospheric conditions. Slight variations in its reaction and chloride concentration were found to correlate simultaneously with changes in the blood.

2. While the composition of the sweat is correlated slightly with that of the blood, the product elaborated by the sweat glands seems to be primarily independent of chemical conditions in the body. The sweat glands are therefore comparable to the salivary glands and mammary glands in their work of producing a uniform secretion. This secretion is more dilute than the blood in respect to most dissolved substances.

3. The compensating activities of the kidneys which correlates their work with that of the sweat glands, is a correlation only on the part of the kidneys, since the kidneys are accustomed to correct the variations in the blood's composition.

4. The rate of sweat production is probably directly proportional to the "effective temperature" above 90°F.

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STUDIES OF THE BRAIN STEM

VII. The Respiratory Exchange and Heat Production after Destruction of the Body Temperature-Regulating Centers of the Thalamus

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It has been shown by several workers (1), (2), (3) that destruction of the optic thalamus leads to the permanent and complete loss of the ability to maintain a normal body temperature in a fluctuating atmospheric temperature. This is not a temporary effect due to surgical or spinal shock for, as shown by Bazett and Penfield (3) working with cats, and by the writer (2) with birds, such animals can be kept alive for periods of two to six weeks without recovering the ability to regulate body temperature, if the body temperature is artificially maintained by keeping the animals in a warm incubator. It is obvious that this loss might be due to a failure of either or both the thermogenic and the thermolytic mechanisms. With reference to heat production after loss of the temperature-regulating centers, Bazett and Penfield noted in their animals that shivering did not occur when they were exposed to cold. Barbour (4) stated that the same is true after high transection of the spinal cord. The writer has observed in a dog with a mid-thoracic spinal transection, that when exposed to cold nine months after the transection, shivering occurred in the shoulder and fore-leg muscles but not in the hind quarters. Birds rendered poikilothermous by transection of the brain-stem just anterior to the mid-brain, do not exhibit the increased muscle activity which might lead to increased heat production. After these cerebral and spinal lesions, atmospheric cold does not therefore stimulate the thermogenic systems as in normal animals.

These facts might be explained on the hypothesis that the thermogenic organs are stimulated reflexly by cold acting on the nerve centers in the optic thalamus. They do not however give any information as to whether the activity of these centers is a necessary condition for the metabolic changes responsible for heat production within the tissues. We have therefore attempted by measurements of the respiratory exchange, before and after the destruction of the optic thalamus, to deter-

mine whether or not the loss of these cerebral nerve centers in itself depresses metabolism and heat production within the body.

For such a determination it is clear that certain well-known factors must be excluded which would alter heat production. The following precautions therefore were taken.

1. Shock. Of all the homothermous animals, spinal shock is at a minimum in birds. Within a few minutes after the cerebral operation all signs of temporary shock effects disappear. Twenty-four hours were allowed for recovery from any temporary effects before the operated birds were used in this work.

2. Infection. As commonly recognized, birds are highly resistant to bacterial infection. After long experimentation with the bird brain, I have never seen any indications of infection following the cerebral lesions.

3. Atmospheric temperature. As is indicated below, exposure of the normal bird to atmospheric cold increases very markedly the rate of oxygen consumption. To control this factor all determinations have been made at atmospheric temperatures of 28° to 30°C., at which the rate of oxygen consumption seems to be the lowest. Above this temperature, the oxygen consumption is increased as the thermolytic respiratory mechanism is called into play. At an atmospheric temperature of 30°C. the poikilothermous decerebrate pigeon exhibits the body temperature of 39° to 40°C. which is the normal value for the pigeon. The difference of 10°C. between the atmospheric temperature and the body temperature is due to the insulating action of the feathers and can be abolished by removal of them. If the feathers of the poikilothermous pigeon be removed the body temperature then sinks to within one or two degrees of the environmental temperature. Metabolic determinations made therefore at the atmospheric temperatures of 28° to 30°C. give the basal values for both the normal pigeon and the poikilothermous pigeon with a body temperature artificially maintained at the normal value of 40°C.

4. Digestive activities. Feeding leads to a tremendous increase in the oxygen consumption (table 1). All comparative determinations therefore have been made with at least twenty-four hours' deprivation of food but with sufficient water. Particular care was taken that all the animals herein described were provided with an ample water supply. The effects therefore are not due to lack of water in the body. Whether or not the distribution of this water within the body of the operated animal was the same as that of the normal animals, cannot be stated.

5. Variations between different animals. It has long been known that the rate of oxygen consumption bears an inverse relation to the size of the body. In small animals of varying size, such as birds, this is so striking that it was found one animal could not be used as a control on

another. We therefore made determinations on each bird before operation and then-after removal of the temperature, regulating centers, repeated the determinations on that same animal.

6. Surgical technique. The method of decerebration and destruction of the optic thalamus, which renders the bird poikilothermous, has been described previously. After this destruction of all cerebral tissue anterior to the mid-brain, many animals fail to survive for more than one or two days. Hemorrhage into the remaining parts of the brain with polyuria and loss of water from the body account for many of these deaths (8). For the metabolic determinations connected with the present

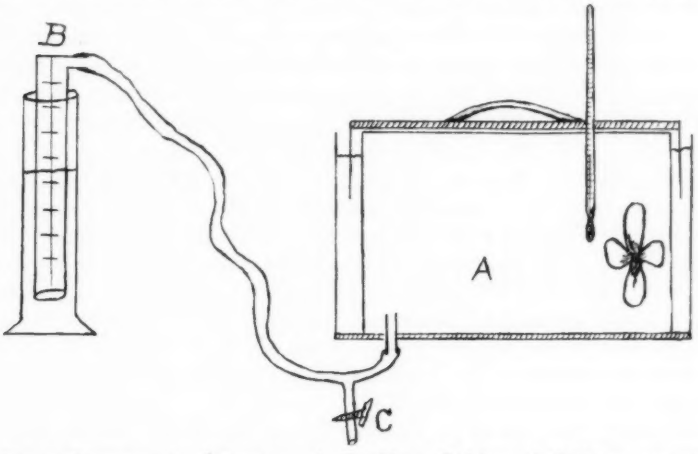

Fig. 1. Diagram of calorimeter. *A*. Double walled box of 15,000 cc. capacity with thermometer and fan. The space between the double walls is filled with water. The top is removable. *B*. Accessory air reservoir floating on water. A measured amount of air may be added to or taken from the calorimeter to maintain a constant pressure of the enclosed air. *C*. Stopcock connecting with sampling tube.

study such animals cannot be used. Only those animals were used which survived the operation for at least seven days. Owing to the surgical difficulties of working at the base of the brain, many metabolic determinations had to be made on animals which did not survive the operations. These are not included in this report.

METHODS. In work previously reported (5) the attempt was made to gather information as to the metabolic rate of the poikilothermous animal by determinations of the carbon dioxide output. The range of variation was so great that the effort was made to provide a better method and one whereby determinations could be made not only of the carbon dioxide but also the oxygen consumption.

Several attempts were made to construct different types of calorimeters for this work, but the one finally employed was as follows (fig. 1). A double walled box was made of galvanized iron. The capacity of the inner chamber was 15,000 cc. The space between the double walls was filled with water at the desired temperature. The top of the calorimeter fitted into the water jacket insuring an air-tight arrangement. Within the chamber was fitted a standardized thermometer and a small electric fan to mix the air uniformly. A small accessory air reservoir of 1000 cc. capacity was connected by tubing with the inner chamber to admit or withdraw such quantities of air as might be needed to maintain constant the volume and pressure of the air in the calorimeter chamber. No light was admitted into the calorimeter and with the bird this usually insures a condition of quiet and muscular inactivity. A stethoscope was fastened to the wall of the chamber through which any movements of the bird could be detected. No determinations were made when the bird was walking or struggling within the box. Samples of air were taken from the chamber for analysis with a standarized Haldane apparatus before and after the bird was enclosed. The bird was allowed to stay in the chamber for intervals of 15 to 25 minutes to permit the carbon dioxide to accumulate to the maximum values of 0.6 to 0.8 per cent. Knowing the total volume of the inner chamber the atmospheric temperature and barometric pressure, the volume of the bird, and the percentages of oxygen and carbon dioxide at the beginning and end of the experiment, the oxygen consumption and the carbon dioxide output may be directly calculated.

Obviously the method of allowing the carbon dioxide to accumulate within the chamber is at fault since it will lead to an increase in the activity of the respiratory muscles depending on the concentration of the gas. According to the work of Scott (6) (cited by Macleod), a concentration of carbon dioxide of 0.8 per cent increases the pulmonary ventilation about 15 per cent. Since in this work the carbon dioxide accumulation concentration at the beginning of the experiment averaged about 0.05 per cent and at its close about 0.60 per cent, with a gradual increase from the lower to the higher value, the error involved would be not greater than 8 to 10 per cent. There is therefore this possible error in the absolute values obtained. However since the same method was used uniformly in all cases, it was thought it would not vitiate the relative values obtained before and after the surgical operation.

All gas analyses were made in duplicate and discarded unless they agreed to within 0.04 per cent. Calculation showed that this margin of error, owing to the short time of the experiment and the small volume of gas in the calorimeter, permits an error of $\pm$ 0.02 in the respiratory quotient. With the hope of minimizing this error all determinations were made twice and the average of the two determinations is given.

Knowing the oxygen consumption and the respiratory quotient, the heat production was calculated from the tables of Zuntz and Schumberg as quoted by Boothby and Sandiford (7). Owing to the fact that the determinations were made for short time intervals only, no correction has been applied for protein and non-protein metabolism. Under the conditions of the experiments the total dry solids of the urinary and intestinal excretions for the normal and operated birds was less than 1 gram per 24 hours. We have not been able as yet to detect any constant differences in the amount of total solids excreted by the normal and poikilothermous birds if both are maintained at the atmospheric temperature of 30°C. and without food. According to Boothby and Sandiford, correction for protein metabolism "rarely makes a difference of 3 per cent in the amount of heat found by neglecting this refinement." Since similar amounts of urinary solids are excreted by both the normal and operated birds it will not alter the relative values obtained.

TABLE 1

Metabolism of the normal adult pigeon, feeding and fasting, when exposed to atmospheric warmth and cold

CONDITIONS	ATMOSPHERIC TEMPERATURE	O_2 PER HOUR	CO_2 PER HOUR	RESPIRATORY QUOTIENT	CALORIES PER HOUR
	°C.	cc.	cc.		
24 hours fasting	29	290	210	72	1.36
48 hours fasting	30	290	212	73	1.36
After feeding	30	429	397	92	2.12
After feeding	29	445	424	95	2.21
After feeding	9	1067	1002	94	5.30
After feeding	15	988	963	97	4.95
24 hours fasting	25	391	273	70	1.83
24 hours fasting	9	542	402	74	2.56

RESULTS. The values obtained for the oxygen consumption and the carbon dioxide output of two pigeons before and after destruction of the cerebral temperature regulating centers are cited in tables 2 and 3.

In the normal fasting pigeon at an atmospheric temperature of 28° to 30°C. the oxygen consumption is from 200 to 300 cc. per hour, the carbon dioxide elimination is from 150 to 230 cc. per hour, with a respiratory quotient of about 0.70. Feeding raises the oxygen consumption to 400 to 450 cc. per hour, the carbon dioxide excretion to about 400 cc. per hour and the respiratory quotient rises to about 0.95. Exposure of the normal feeding bird to the atmospheric cold of 9° to 15°C. raised the oxygen consumption to 980 to 1050 cc. per hour, the carbon dioxide excretion to 935 to 1000 cc. per hour and the respiratory quotient to about 0.96.

For comparing the metabolic rates of the bird before and after reduction to the poikilothermous condition, determinations were therefore made at

TABLE 2

Metabolism of adult pigeon 38 before and after destruction of the thalamus. Atmospheric temperature 28° to 30°C. and body temperature 39° to 41°C.

CONDITIONS	BODY WEIGHT	O_2 PER HOUR	CO_2 PER HOUR	RESPIRATORY QUOTIENT	CALORIES PER HOUR
	grams	*cc.*	*cc.*		
24 hours fasting	325	289	208	0.72	1.36
48 hours fasting	315	298	217	0.72	1.40
72 hours fasting	312	284	195	0.69	1.33
96 hours fasting	308	258	180	0.70	1.21
120 hours fasting	304	218	155	0.71	1.02
144 hours fasting	300	217	160	0.73	1.02
Ten days abundant feeding and then optic thalamus destroyed					
24 hours fasting	298	262	172	0.66	1.20
48 hours fasting	290	251	178	0.71	1.18
72 hours fasting	292	200	148	0.74	0.95
120 hours fasting	280	222	157	0.71	1.04

TABLE 3

Metabolism of adult pigeon 39 before and after destruction of the thalamus. Atmospheric temperature 28° to 30°C. and body temperature 39°to 41° C.

CONDITIONS	BODY WEIGHT	O_2 PER HOUR	CO_2 PER HOUR	RESPIRATORY QUOTIENT	CALORIES PER HOUR
	grams	*cc.*	*cc.*		
24 hours fasting	352	290	212	0.73	1.36
48 hours fasting	349	327	227	0.69	1.52
72 hours fasting	350	345	229	0.66	1.58
120 hours fasting	340	222	158	0.71	1.04
21 days abundant feeding and then the thalamus destroyed					
72 hours fasting	281	366	254	0.69	1.71
96 hours fasting	275	260	181	0.70	1.22

TABLE 4

Metabolism of pigeon 38 after destruction of the thalamus. Varying body temperatures. Forty-eight hours fasting

ATMOSPHERIC TEMPERATURE	BODY TEMPERATURE	O_2 PER HOUR	CO_2 PER HOUR	RESPIRATORY QUOTIENT	CALORIES PER HOUR
°C.	°C.	*cc.*	*cc.*		
29	39	251	178	71	1.17
25	35	178	132	74	0.84
21	32	107	87	81	0.51

the standard atmospheric temperatures of 28° to 30°C. Furthermore since the necessary cerebral operation frequently leads to gastric disturbances, the comparative determinations were made while the animals were in the fasting condition. This is a minor consideration in the case of the determinations herewith included as the animals selected for this work were ones which exhibited no marked digestive disorders after the cerebral ablations.

The values of the oxygen consumption, carbon dioxide excretion, respiratory quotient and calculated heat production before and after destruction of the temperature centers are given in tables 2 and 3. It will be noted from these tables that within the range of 10 per cent error in the determinations the loss of the cerebral hemispheres and the optic thalamus which destroys the ability to regulate body temperature does not appreciably depress the basal metabolism or the heat production within the body, if the body temperature is artificially maintained at the normal level of about 40°C.

By a comparison of tables 1 and 4 it will be noted that in the normal bird, exposure to atmospheric cold leads to an increase in the heat production and maintenance of a normal temperature, but after destruction of the thalamus the heat production and the body temperature are both lowered as the atmospheric temperature is lowered. Specificially therefore one of the effects of destruction of the thalamus is the loss of ability to increase the heat production in response to lowering the atmospheric temperature below 30°C. The respiratory quotient is of similar value in the normal pigeon and in the poikilothermous pigeon provided the atmospheric temperature, body temperature and fasting conditions are similar in both animals. This indicates therefore that within the limits of error in the methods employed there is no gross alteration of the quality of metabolism following the removal of the temperature regulating centers. The loss of ability to regulate body temperature after destruction of the thalamus cannot then be attributed to any direct influence exerted on the metabolism of the tissues.

In previous work (5) it has been pointed out that after destruction of the thalamus there is interference with the thermolytic mechanisms. The normal response of the bird to increasing atmospheric temperature is first the reduction of heat production by muscular inactivity, and secondly, the appearance of polypnea with increased loss of heat by evaporation of water from the respiratory tract. After loss of the thalamus polypnea does not appear when the body temperature rises above the normal value of 42°C.

The preceding considerations seem therefore to indicate that the mode of action of the thalamic temperature regulating centers is not by any direct influence on tissue metabolism but is exerted through the ordinary

channels of nervous stimulation or lack of stimulation of muscular activity. It seems to us that this is not contradictory of the important work recently done on the relations of the water balance of the body and temperature regulation, but it suggests that the changes of water distribution in response to atmospheric temperature changes, so far as muscle tissue is concerned, are secondary to the changes of muscle activity which is directly influenced by the nervous system.

SUMMARY

The oxygen consumption, carbon dioxide elimination and heat production were determined in pigeons before and after destroying the nerve centers of the optic thalamus which are essential for the regulation of body temperature.

Under standard conditions of fasting and of maintenance at an atmospheric temperature of 28° to 30°C. no appreciable difference was found between the basal metabolism and the heat production values of the homothermous and the poikilothermous conditions.

The animal with thalamus intact increases its heat production when exposed to atmospheric cold; in the absence of the optic thalamus the heat production falls as the atmospheric and body temperatures fall.

Birds in which the thalamus has been destroyed do not respond to atmospheric cold by increased activity of the striated muscles such as shivering, walking or flying.

Since activity of the striated musculature is a major source of heat production in the body; since increased activity of this musculature does not appear when the animal is exposed to atmospheric cold if the thalamus has been destroyed; and since determinations of basal metabolism indicate no disturbance of the heat producing chemical activities of the tissues whether thalamus be present or not, it is suggested that when the thalamus has been destroyed, pathways of reflex discharge have been broken which, commonly stimulated by cold, lead to increased muscular activity and heat production.

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A STUDY OF THE RABBIT AS A TEST ANIMAL FOR DETERMINING THE POTENCY OF INSULIN PREPARATIONS

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An adequate method for testing the potency of various insulin preparations is of paramount importance to laboratory workers who are interested in comparing the action of different products and of no less importance, as a safeguard, to those who are interested in the clinical use of these products. Clinical experience with anti-diabetic preparations made from the pancreas has shown that they form no exception to clinical experience with other drugs in the matter of variations in the response shown by different individuals. This, again, makes it essential that we have some method of standardizing these products—indeed it makes standardization second only in importance to efficient methods of preparation.

The ideal method of determining the amount of anti-diabetic substance obtained from the pancreas would be that of weighing the dried, pure product. This undoubtedly will be possible in time. Even when this is accomplished it will still be necessary to standardize products biologically in order to determine dosage for clinical use. Next to weighing the pure product a chemical method of assay would be desired but none is at hand at present. But here, again, biological testing would ultimately be needed.

The most important step in the direction of devising an adequate method for biological testing of anti-diabetic preparations was taken by the Toronto workers when they introduced the idea of using normal rabbits for test animals.

The first thought in regard to standardization at the Toronto laboratory is embodied in the following quotation: (1) "As a tentative basis for the physiological assay of insulin we consider as one unit the number of cubic centimeters which causes the blood sugar of normal rabbits to fall to 0.045 per cent within four hours." Later the appearance of convulsions, together with a fall in blood sugar below this level, was used. Still later another line of investigation was undertaken when the attempt was made to control by insulin the artificial hyperglycemia produced

by the injection of epinephrin (2). This procedure proved to offer difficulties as is shown in the following statement: "Taking these results as a whole it is plain that much more work must be done before it can be told to what extent insulin can antidote the adrenalin effect. We are hopeful that it may be possible to determine the dosage of insulin in terms of the amount capable of antidoting the effect of a standard dose of adrenalin." It would seem that the procedure would be objectionable in that it introduces at least two more variables (accurate biological standardization of adrenalin, and animal variation in reaction to adrenalin) in addition to the difficulty experienced from animal variation in reaction to insulin. In the last extensive study of this problem (5) the conclusion was reached that the method offers no practical advantage over the one previously employed.

During the first year of experimental work at this laboratory diabetic dogs were used as the test animals for the various products which were made. There are several objections to the use of these animals, the chief being that considerable surgical skill is required to depancreatize these animals successfully, and another being that they are expensive and somewhat difficult to obtain, and finally that it is difficult to keep on hand a large enough number to take care of any volume of experimental work. Moreover, it has been pointed out that depancreatized dogs react more markedly to anti-diabetic preparations than do normal animals so that unless the former could be kept under strictly comparable conditions it would be difficult to draw conclusions valid for standardization purposes. It is always difficult to determine standards for normal animals and the difficulty becomes unsurmountable in the case of pathological animals.

About eleven months ago, following the suggestion made by the Toronto workers, we began using normal rabbits for our test animals and have been doing so since that time. Others who have used rabbits for this purpose have apparently met with the same difficulties in regard to animal variations that have troubled us. That the Toronto workers have not found normal rabbits ideal for standardization purposes is admitted in their statement (1): "The failure . . . . of preparations that were of considerable potency in one animal to cause any hypoglycemia in others is obviously unsatisfactory." In this paper we have attempted to gather some of the data obtained from our experiments upon rabbits for the purpose of determining the conditions under which the rabbit can be used for standardizing anti-diabetic preparations.

Preparation for tests. The rabbits used for testing our products were animals weighing with few exceptions 2 kilos or more. The actual weight of the animals must be carefully checked from time to time owing to the fact that animals in active use show great variations in weight. Under

ideal conditions we feel that a rabbit should not be used for testing purposes oftener than twice in a week, allowing at least two days of rest between consecutive tests and a longer interval for those rabbits in which convulsions occur. The most important factor is the degree of reaction to the products, i.e., the absolute blood-sugar drop which is produced. Not more than five consecutive tests should be made upon an animal without re-weighing. A variation of half a kilo in a week, in animals receiving potent preparations, is common. Occasionally an animal is found which maintains practically constant weight over a long period of time in spite of the fact that it received potent products. The ideal method of watching the weight is to weigh the animals each time they are used, and under no conditions should the weight be neglected.

Every attempt should be made to keep the animals in good condition and normal for tests. Our animals are regularly given all the chopped carrots and oats which they desire.

Preparatory to testing, the animals have been without food for eighteen hours.

Blood taking. Blood is taken from the ear veins. Before this is done the ear is briskly rubbed a few times to promote vasodilatation. Occasionally (during cold weather) it has been found of advantage to rub the ear gently a few times with a piece of cotton saturated with xylol.

This acts as an irritant and often makes it possible to obtain blood from animals which otherwise would offer great difficulty. As soon as vasodilatation occurs the xylol is wiped off with a piece of cotton saturated with ethyl alcohol. The ear is always wiped with alcohol whether xylol is used or not.

Venepuncture is done with a needle sterilized with alcohol. The marginal vein is used whenever possible; otherwise one of the central group is used. Blood (expressed by milking the base of the ear) is allowed to drip into a test tube containing a small amount of finely powdered potassium oxalate. The blood is kept agitated during the entire time of collection by tapping the side of the test tube with the finger. Immediately after taking the first blood sample, the product which is to be tested is injected subcutaneously and the time of injection noted. Two hours after the injection a second blood sample is taken and the difference between the two blood-sugar readings is found and used (as explained later) for determining the number of rabbit units per kilo of pancreas used. Although we know that in some cases the maximum drop in blood sugar occurs later than two hours after the injection yet we feel that the vast majority of potent preparations produce their effect within this interval. Table 1 shows a few typical instances of this. In the last three cases in the table (animals D-3, F-2 and F-6) the animals reached their lowest levels within an hour and

maintained the low level for three hours. In the case of D-1 the maximum occurred in an hour and a half. The two-hour interval, then, has seemed to be the best for these tests.

Injections. All products have been tested by subcutaneous administration. The injections have been made under the skin of the back on either side of the spinal column. Before injection the areas selected are denuded of fur and sterilized with tincture of iodine. An infection at

TABLE 1

Showing time of maximum drop in blood sugar

ANIMAL	TIME	BLOOD SUGAR (MGM.)	FALL (MGM.)	TIME OF MAXIMUM FALL
D-1	10:29 a.m.	110		
4/27/23	10:30	4 cc. #125 By		
	½ hr. 11:02	108	2	
	1 hr. 11:34	90	20	
	1½ hr. 12:04 p.m.	71	41	1½ hr.
	2 hr. 12:30	81	29	
	2½ hr. 1:04	83	27	
	3 hr. 1:33	89	21	
D-3	9:40 a.m.	118		
4 28 23	9:44	40 cc. #129-A		
	1 hr. 10:46	83	35	1 hr.
	2 hr. 11:48	83	35	
	3 hr. 12:50 p.m.	84	34	
F-2	11:16 a.m.	113		
5/22/23	11:20	40.8 cc. #144		
	1 hr. 12:30 p.m.	71	42	1 hr.
	2 hr. 1:21	71	42	
	3 hr. 2:20	76	35	
F-6	11:08 a.m.	107		
5/22/23	11:10	7.5 cc. #136c		
	1 hr. 12:26	56	51	1 hr.
	2 hr. 1:18	63	44	
	3 hr. 2:16	63	44	

the site of injection has not been seen among our animals. In those cases in which it has been necessary to give large amounts of dilute products it has been customary to inject at several different points. Not over 10 cc. are injected at one site.

Blood-sugar determinations. All blood-sugar determinations have been made by the Folin-Wu (8) method. As soon as possible after collection of the samples the bloods are precipitated and the filtrates are all read together, later in the day, against the same standards.

TABLE 2

Blood-sugar levels of normal rabbits

OBSERVERS	NUMBER OF DETERMINATIONS	NUMBER OF ANIMALS	PRELIMINARY FASTING PERIOD (HRS.)	LOWEST READING OF SERIES (MGM.)	HIGHEST READING OF SERIES (MGM.)	MEAN	MEDIAN	STANDARD DEVIATION (MGM.)	PER CENT WITHIN STANDARD DEVIATION	PROBABLE ERROR (MGM.)	PER CENT WITHIN PROBABLE ERROR	COEFFICIENT OF VARIABILITY	BLOOD SUGAR METHOD
Scott and Ford	85	27	24	68	177	118	116	21.5	68	14.5*	82*	18.2*	Maclean (7)
University of Toronto	157	?	Few	83	154	116	115	12.2	69	8.2	66	10.5*	Shaffer and Hartman (6)
University of Rochester	100	100	18	71	143	105	104	17.0	72	11.4	49	16.1	Folin-Wu (8)

* Our calculations.

TABLE 3

Blood-sugar levels of rabbits used for testing anti-diabetic preparations

OBSERVERS	NUMBER OF DETERMINATIONS	NUMBER OF ANIMALS	PRELIMINARY FASTING (HRS.)	LOWEST READING OF SERIES (MGM.)	HIGHEST READING OF SERIES (MGM.)	MEAN	MEDIAN	STANDARD DEVIATION (MGM.)	PER CENT WITHIN STANDARD DEVIATION	PROBABLE ERROR (MGM.)	PER CENT WITHIN PROBABLE ERROR	COEFFICIENT OF VARIABILITY	BLOOD SUGAR METHOD
University of Toronto	88*	88*	24*	89*	180*	127*	127*	17.2*	74*	11.6*	55*	13.5*	Shaffer and Hartman (6)
University of Rochester	764	64	18	63	182	113	113	17.8	70	12.0	52	15.7	Folin Wu (8)

*Our calculations.

Normal blood-sugar level. The first problem which presented itself in connection with the use of rabbits for test animals was that of determining the normal blood-sugar level for our animals and the allowable range of variation. We are fortunate in having at our disposal two similar studies made by Scott and Ford (4) and G. S. Eadie (3). In regard to the feeding of the animals no essential difference exists—Scott and Ford fed their rabbits once daily on a mixture of grain and green vegetables, usually oats and cabbage; Eadie's rabbits were fed upon oats, and our rabbits were fed upon oats and carrots. There are two respects in which the series shown in table 2 vary—the length of the preliminary fasting period, and the methods of blood analysis employed. The Toronto series corresponds almost exactly to that of Scott and Ford in

TABLE 4

Showing group variations in blood-sugar levels before and after use

ANIMAL NUMBER	ORIGINAL BLOOD SUGAR READINGS (MGM.)	NUMBER OF READINGS	MEAN OF ALL READINGS BEFORE TREATMENT (MGM.)
P-1	107	25	117
P-2	100	39	128
P-3	121	29	120
P-4	110	29	120
P-5	133	41	127
P-6	121	17	120
Mean.....	115	Mean.....	122
H-1	71	8	85
H-2	90	15	91
H-3	102	4	91
H-4	80	14	79
H-5	81	6	84
H-6	83	6	79
Mean.....	84	Mean.....	85

regard to the mean (116 and 118, respectively) so the preliminary fasting period does not appear to have influenced those two series. Comparison of the blood-sugar methods used at the various laboratories has been made by several investigators (4), (6), and has shown that no essential difference exists in the results obtained by them.

Assuming that the conditions are comparable in the three series the only essential difference seen in table 2 is the lower mean obtained for our rabbits. Our standard deviation from the mean is lower than Scott and Ford's but higher than Eadie's. Likewise the probable error and the coefficient of variability, which are functions of the standard deviation, show the same ratios for the three series. The average mean for

the three series is 113 mgm. and the average standard deviation from the mean is 16.9 mgm. giving a range of 96 mgm. to 130 mgm. for normal animals, which have not been used for experimental purposes. It has been somewhat puzzling to explain the low mean which was obtained for our series—105 mgm. as against 116 and 118 mgm. for the other two series. It has occurred to us that perhaps the larger number of animals observed may have some bearing upon this. Scott and Ford made observations upon twenty-seven different animals and we made obsevations upon a group of one hundred animals. We do not know the number of animals in the Toronto series. That great differences exist between different lots of animals is shown by table 4. Here we have tabulated results from one of our best series (P-1 to P-6) and one of our poorest series (H-1 to H-6). There is a difference of 31 mgm. between the means of the two series and the average for these two is 100 mgm. It is clear that if enough animals of the type of the H-series are included in a study the mean blood-sugar level will tend to be lowered considerably. That these animals ran true to form is shown by the fact that the mean level remained the same at the end of the period of use of the animals.

Blood-sugar levels for animals under experimental conditions. For the purpose of studying the effect of repeated injections of insulin preparations for testing purposes upon the blood-sugar levels of rabbits we selected the initial blood-sugar readings of our experimental animals. There were 764 readings representing 64 different animals in the group studied. In table 3 the results are shown. The mean level of all of the readings is 113 mgm. and the standard deviation from the mean is 17.8 mgm. Comparing this with the normals shown in table 2 it will be seen that the standard deviation, probable error, and coefficient of variability are practically the same for the two groups. The only noticeable difference between the two groups is in the mean, which is 8 mgm. higher for the experimental group.

In order to have a group with which to compare our experimental series we calculated the mean, standard deviation from the mean, etc., for the animals reported in the tables of the Toronto investigators in September, 1922 (1). These results are shown in the first line of table 3. Comparing these with the figures given for their normal animals in table 2 it will be noted that the mean is higher in the experimental series (127 mgm. versus 116 mgm.). The standard deviation is also higher—17.2 mgm. as compared with 12.2 mgm. and consequently the probable error and the coefficient of variability are higher. It is an interesting fact that the mean for each of the experimental series is higher than the mean for the corresponding series of normal animals. This same fact is noticeable in table 4 in which the readings of two

groups of animals are shown before and after use. Since this same increase in the mean of animals under experimental conditions is noted in both the Rochester and the Toronto series, it seems possible that this may represent a rebound on the part of the rabbits' blood following insulin injections.

Significance of high blood-sugar readings. In the case of high levels at the start we have felt that in all probability we have been dealing with an artificial hyperglycemia induced by adrenal stimulation from fright or pain. Every effort is made to eliminate both of these factors and we always make note of any obvious fright or restlessness on the part of the animal during the taking of the blood sample. Theoretically

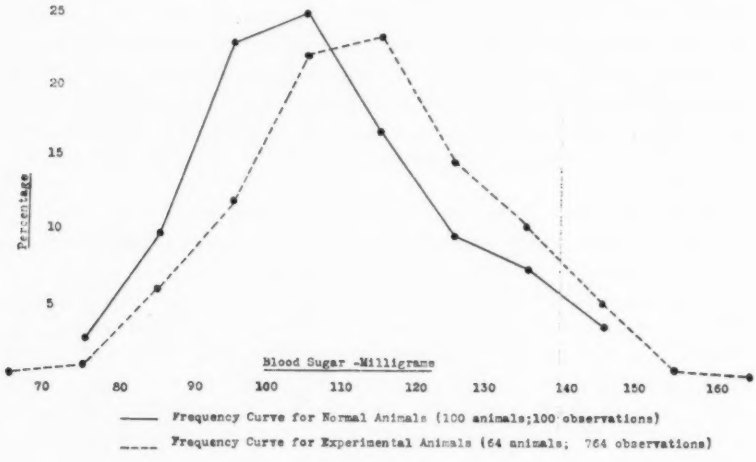

Fig. 1

older animals, i.e., animals in use for a longer time, would be less likely to show such reactions. A study of the successive levels of a given animal shows no progressive lowering of level coincident with longer use of the animal so that we are inclined to feel that under ordinary conditions we need not consider these factors. That the possibility of inducing an artificially high level exists is shown by the observation that in a few cases in which animals have been frightened levels above 0.200 and even 0.300 have been obtained. Scott and Ford (4) point out the fact that fright is the most common emotion in rabbits and that fright is usually (though not always) accompanied by hyperglycemia.

Significance of low blood-sugar readings. Blood-sugar determinations which are much below the limit allowable for animal variations (0.088)

indicate that the animals are unfit for testing purposes. All tests made upon animals with abnormally low initial readings should be discounted. So far as our readings have shown, there is no typical ante-mortem change in blood-sugar level. However, we have observed several times a progressive fall in the blood-sugar level for one or two days before death. These observations are shown in table 5. The six instances recorded in

TABLE 5

Progressive fall in initial blood-sugar level

ANIMAL NUMBER	BLOOD-SUGAR LEVEL AV. GM.	DATE	TIME	1ST BLOOD SUGAR	B.S. 2 HRS. AFTER INJECTION	DIFF.	REMARKS
B-6	0.128	4/18/23	11:55 a.m.	0.115	0.031	−0.084	
		4/19/23	10:54 a.m.	0.059	0.009	−0.050	
			1:00 p.m.				Dying
			4:00 p.m.				Dead
C-4	0.108	4/ 9/23	11.17 a.m.	0.114	0.043	−0.071	
		4 11/23	11.05 a.m.	0.053	0.032	−0.021	
			5.00 p.m.				Found dead
P-5	0.127	5/ 2/23	11:18 a.m.	0.103	0.038	−0.065	
		5/ 3/23	9:52 a.m.	0.098	0.038	−0.060	
		5/ 4/23	8:30 a.m.	0.072	0.038	−0.034	
		5/ 5/23	8:00 a.m.				Found dead
A-4	0.107	5/ 6/23	10:27 a.m.	0.113	0.033	−0.080	
		5/ 9/23	10:51 a.m.	0.085	0.023	−0.062	
		5/10/23	10:00 a.m.	0.066	0.084	+0.018	
			2:10 p.m.				Weak
		5/13/23	8:00 a.m.				Found dead
C-3	0.113	5/ 2/23	11:25 a.m.	0.107	0.036	−0.071	
		5/ 3/23	10:27 a.m.	0.089	0.026	−0.063	Convulsions
		5/ 4/23	8:59 a.m.	0.032	0.030	−0.002	
		5/ 5/23	8:00 a.m.				Found dead
D-6	0.117	5/ 2/23	12:00 m.	0.115	0.031	−0.084	
		5/ 4/23	9:37 a.m.	0.072	0.075	+0.003	
		5/ 5/23	8:00 a.m.				Found dead

this table are the only ones found among the 64 animals studied. With rabbit C-4 it will be noted that two days after the animal received a preparation which caused a fall of 0.071 gram the initial blood-sugar level was far below normal. The same thing is shown with rabbit D-6 of this table. In the case of rabbit A-4 it will be seen that the initial blood-sugar level was far below the average level for the animal *three days* after a drop of 0.080 mgm. These observations show conclusively

the folly of employing the same animals for testing purposes without allowing sufficient time to elapse between tests for the animals to recuperate fully. In all of these groups of tests the animals show a progressive lowering of the initial daily levels as a result of prolonged and cumulative action of the preparations tested, with ultimate loss of the animals regardless of repeated glucose injections. Moreover, in all of these cases no reliance can be placed upon any of the tests made after the first day recorded in the groups of tests.

New lots of animals show great variability in regard to their normal levels and our experience with different lots fully bears out our other observations in regard to the serious significance of low initial readings. Recently we received a half-dozen animals which showed persistently low initial blood-sugar levels. Nearly all of these animals died after less than two weeks of use and some died after as few as two tests. On the other hand, those animals showing persistently high normal levels have been the ones which have lived longest under testing conditions. Two typical lots of animals are shown in table 4. In the case of the P-series 180 observations were made upon the six animals and only 53 observations were made upon the H-series. The same general types of preparations were tested upon the two series.

Convulsions; convulsion level. When rabbits are given potent preparations, convulsions frequently occur. The appearance of convulsions and their relief, at times, by glucose injections, constitute the most striking phenomena encountered in this work. During the prodromal stage the rabbits are seen to be lethargic and tests have shown that during this period (when the animal appears to develop a curious stare) there is visual impairment. During this stage the animal seems to develop a hypersensitiveness to tactile stimuli—in many respects it resembles a strychninized animal—so that the slightest touch suffices to initiate a convulsion. The animal often starts to run, quite unconscious of obstacles in its path (loss of vision?) usually in one direction—showing a definite ataxia to the right or to the left—describing circles. If it falls the animal usually rolls in one direction continuously, meantime having clonic contractions of the body and leg muscles. During the convulsive stage these animals appear to be anesthetic—they show no evidence of pain when the ear is pricked or when injections are made. The cornea is not sensitive at this time. Some animals have been seen to jump straight into the air to a height of 2 feet when touched; others appear to be in profound shock and lie on one side and exhibit the clonic contractions mentioned above. Occasionally a complete opisthotonus has been seen and the animals have squealed loudly. In some cases the convulsions are precipitated by the animal suddenly falling over. During the convulsions it is difficult, in fact often impossible, to obtain

blood from the ear veins (vasoconstriction?). Delayed convulsions occur rather frequently and probably many deaths are due to untreated convulsions occurring during the night for animals showing marked response to certain preparations are often found dead the following morning.

When an animal is found in convulsions subcutaneous injections of 10 cc. of a 25 per cent glucose solution are made. Sometimes the animal which but a moment before showed symptoms almost suggestive of furious or convulsive rabies, is restored in a few minutes (as if by magic!) by a single injection of 10 to 20 cc. of glucose solution and

TABLE 6

Convulsion level

(All readings of 0.045 or below)

BLOOD-SUGAR RANGE	CONVULSIONS		TOTAL NUMBER OF OBSERVATIONS
	Seen	Not seen	
0.000 to 0.005	1	1	2
0.006 to 0.010	2	0	2
0.011 to 0.015	9	2	11
0.016 to 0.020	5	3	8
0.021 to 0.025	16	7	23
0.026 to 0.030	12	13	25
0.031 to 0.035	5	25	30
0.036 to 0.040	6	33	39
0.041 to 0.045	2	26	28
Total.....	58	110	168

Mean = 25 mgm.
Median = 24 mgm.
St. Dev. = 9.2 mgm.
Probable error = 6.2 mgm.
Per cent in St. Dev. = 66 (0.016–0.034)
Per cent in Pr. Error = 48 (0.019–0.031).

promptly begins eating. We have not found it possible, however, to restore all animals by glucose injections and have often observed that when they have been restored they show no tendency whatever to eat. Some animals, on the other hand, seem almost to have an instinctive realization that they need sugar and begin nibbling carrots the moment they are brought out of the convulsive state.

We have recently adopted the routine procedure of giving 50 cc. of a 20 per cent glucose solution by stomach tube to all animals used for testing preparations. This is done immediately after the second blood has been taken.

Because of the striking character of convulsions there is a strong temptation to use the appearance of convulsions, in some way, as a criterion of potency. With this thought in mind we recorded all instances in which the blood sugar was reduced to 0.045 gram or lower. These results are shown in table 6. In this table we have indicated the various observed levels from zero to 0.045 and in parallel columns those instances in which convulsions were seen and those in which convulsions were not seen. In a total of 168 observations at or below 0.045 gram convulsions occurred in 58 cases or 34.5 per cent. The mean convulsion level for those animals in which convulsions were seen is 0.025 gram with a standard deviation of 6.2 mgm. This gives a range from 0.016 gram to 0.034 gram within which most of the convulsions in our animals occurred.

The highest level at which a convulsion has been seen among our rabbits is 0.066 gram and the lowest level at which a convulsion was not seen is 0.003 gram. This shows that the convulsion level is not *per se* a definite point. Most of the phenomena connected with convulsions are not understood. One factor seems to be the low level of blood sugar but we are convinced that an equally important factor is the suddenness with which this low level is brought about. We have occasionally found animals, apparently normal, with an initial level at or below the average convulsion level. Rabbit G-3 on May 31, 1923, was found to have a level of 0.026 gram and appeared normal in all respects—in fact it had had no treatment during the previous five days (having previously been used but once and then for testing a product of moderate potency) and should have been normal. Again, animal H-6 was found on June 4, 1923, to have a level of 0.013 gram with no evidence of abnormality. All of our observations on convulsions make us feel that they are incidental results of certain *at present* unknown combinations of factors and have no definite significance in regard to potency of preparations. The probability of the production of convulsions by a given product increases in proportion to the lower initial blood-sugar levels of animals. In other words, we feel that it is the result of the sudden production of *an absolute fall of a certain magnitude.*

Absolute blood-sugar drop. For the purpose of determining the potency of preparations made in this laboratory we have used since January, 1923, an absolute blood-sugar drop of 0.070 gram in a 2-kilo rabbit, during a time interval of two hours, as a "rabbit unit." On the basis of the number of grams of pancreas represented by the amount of product needed to produce a rabbit unit we have calculated the number of rabbit units per kilo of pancreas represented by that particular product. All products have been reduced to this common basis and

comparisons of different preparations are made on the basis of the number of rabbit units per kilo which they represent.

The following formula may be used to simplify the calculation. $14.28 \frac{a}{R}$ where a is the number of milligrams fall in blood sugar in two hours and R the number of grams of pancreas represented by the dose for a two-kilo rabbit. It is a contraction of the longer formula $\left(1000 \left(\frac{a}{70} = \frac{x}{w}\right)\right.$, where $\frac{x}{w}$ is the ratio mentioned. All the calculations of total units in the tables which follow have been made by this formula.

The use of the appearance of convulsions seems to us to be a less reliable index of potency than the determination of the actual fall in blood sugar, for two reasons: first, that animals show great variability in the level at which convulsions occur; and, secondly, that the normal levels vary within such wide limits. A preparation causing a uniform drop of 0.070 gram would be likely to produce convulsions in an animal starting at a level of 0.090 gram, whereas in one starting at a level of 0.130 it would cause a reduction to a point considerably above the usual convulsion level (as shown in table 6). Moreover, we believe that under strictly normal conditions products cause absolute drops in the blood sugar. In the succeeding tables we shall show the evidence which supports this conclusion.

Optimum dosage. We have adopted the practice of giving the smallest amount of a product which suffices to give a rabbit unit drop (0.070 gram) or less. This is the maximal drop which can be allowed for normal animals for the reason that if a greater drop is produced the lower readings fall in a range in which colorimetric determinations are very inaccurate. In very potent preparations as little as 2.5 grams of pancreas often represents a rabbit unit and an error of 0.010 gram in the colorimetric determination makes a difference of 57 rabbit units in the final calculation based upon this pancreas ratio. Because of the unavoidable rather large biological variation among rabbits even under conditions as nearly standardized as is possible we have felt that a blood-sugar drop well beyond the range of animal variation should be the one adopted for standardization.[1]

[1] The following statement from Scott and Ford (4) supports this view. "One could not safely state that a change in the glycemia, observed during the course of an experiment, was due to the imposed experimental conditions unless it differed from the mean of the control by more than twice the standard deviation, or better, by the sum of the standard deviations of the control series and of a series of animals subject to conditions similar to those of the experiment. That is, should the range of standard deviation of an experimental series overlap that of the normal series or at most overlap it to any considerable extent, general conclusions could not be drawn, though a modification in a certain direction may occur in a certain proportion of the cases examined."

TABLE 7

Duplicate tests made upon different animals. Blood sugar drop variable—showing animal variations

GROUP NUMBER AND PREPARATION	DATE (1923)	AGE (DAYS)	EQUIV. GM. PAN.	PAN.: WT. RATIO	ANIMAL NUMBER	WEIGHT (KILOS)	1ST BLOOD	2ND BLOOD	DIFF.	CONVULSION TIME	RABBIT UNITS
I. (39)	5/15	6	5.00	5:2K	D-3	2.00	0.081	0.046	−0.035		100
	5/18	9	5.00	5:2K	D-4	2.00	0.085	0.014	−0.071	2 hr. 45 min.	202
II. (40-B)	5/21	6	3.08	2.5:2K	F-3	2.40	0.096	0.067	−0.029		165
	5/24	9	2.50	2.5:2K	H-3	2.00	0.081	0.036	−0.045		256
	6/1	17	2.40	2.5:2K	H-4	1.94	0.068	0.019	−0.049		280
	5/16	1	3.34	2.5:2K	E-4	2.60	0.093	0.023	−0.070	2 hr. 5 min.	400
	5/17	2	3.08	2.5:2K	F-3	2.40	0.108	0.017	−0.091	1 hr. 50 min.	521
III. (41-B)	5/25	4	2.50	2.5:2K	I-2	2.00	0.095	0.080	−0.015		84
	5/25	4	2.50	2.5:2K	G-1	2.00	0.086	0.037	−0.049		280
IV. (41-C)	5/29	8	3.24	2.5:2K	I-2	2.60	0.089	0.065	−0.042		137
	6/4	14	2.42	2.5:2K	H-4	1.94	0.058	0.016	−0.042	2 hr. 10 min.	240
	6/6	16	2.50	2.5:2K	G-1	2.00	0.103	0.044	−0.059		336
V. (41-B + B-P)	5/28	7	2.50	2.5:2K	I-2	2.00	0.121	0.064	−0.057		326
	6/8	18	2.97	2.5:2K	I-4	2.50	0.100	0.034	−0.066		377
	6/1	11	2.73	2.5:2K	H-6	2.20	0.090	0.010	−0.080	2 hr.	457
	6/11	21	2.70	2.5:2K	K-3	2.16	0.113	0.026	−0.087	3 hrs. 27 min.	497
VI. (4-A)	6/9	9	2.97	2.5:2K	J-1	2.38	0.097	0.047	−0.050		286
	6/2	2	2.28	2.5:2K	H-5	1.82	0.096	0.027	−0.069	2 hr.	393
VII. (39-P-3-p)	5/23	22	5.00	5:2K	G-3	2.00	0.105	0.073	−0.032		92
	6/5	14	5.00	5:2K	G-3	2.00	0.097	0.050	−0.047		134

VIII. (6)	6/15	10	2.55	2.5:2K	G-3	2.04	0.077	0.051	−0.026		148
	6/9	4	2.70	2.5:2K	I-6	2.16	0.091	0.049	−0.042		240
	6/13	8	2.85	2.5:2K	J-2	2.28	0.115	0.049	−0.066		377
IX. (7-A)	6/12	6	2.55	2.5:2K	G-1	2.04	0.090	0.038	−0.052	2 hr. 20 min.	297
	6/14	8	2.70	2.5:2K	L-6	2.16	0.114	0.037	−0.077		440
X. (8)	6/16	5	2.98	2.5:2K	K-6	2.38	0.100	0.054	−0.046		263
	6/12	1	2.98	2.5:2K	I-4	2.38	0.108	0.039	−0.069		395

Animal variations. That human beings show great individual variations in their response to drugs is known by every one. We have found that the same truth holds in regard to the response of rabbits to insulin preparations. For the purpose of illustrating the possible range of animal variation and also for the purpose of emphasizing the absolute necessity of eliminating the question of animal variation before conclusions based upon the character of the blood-sugar curve are drawn in regard to the potency of preparations, we have brought together some of our results in table 7. The tests made upon the separate preparations are included under separate group numbers. Checked results have been obtained for each of these preparations.

In group I the product was given to two different animals of the same weight with a variation of 51 per cent from the best yield shown. The only variable factor is the age of the preparation—a factor which other groups show to be unimportant.

In group II five tests are reported upon a single preparation with a variation of 68 per cent from the best yield shown. In two cases convulsions were produced.

In group III the tests were made upon animals of exactly the same weight, fifteen minutes apart, with a variation of 70 per cent from the best yield. Here the age factor is identical.

In group IV there is a variation of 60 per cent in the yield and in the intermediate test convulsions were produced.

In group V four tests were made upon the same preparation with a maximum variation of 35 per cent from the best yield. Convulsions occurred in two cases and it will be noted that the tests which check best were made ten days apart, the latter when the product was twenty-one days old.

In group VII the preparation was tested upon the same animal on two occasions eight days apart with a variation of 32 per cent from the best yield.

It is difficult to account for many of the variations seen among the test animals. We have never seen any evidence of true delay in absorption of products as judged by persistence of the fluid at the site of injection (even in the case of large doses). That there is often a delay in the response to some products seems evident. In the case of one of our products (perfusate 39) three different tests resulted in the death of the rabbits over night although some of the two-hour blood-sugar drops were not sufficiently great to lead us to expect such an outcome. We thought of the possibility of some toxic action of the product but dismissed the idea when later we retested the product without fatal results. Again, recently in testing another product (perfusate 43) we have found the animals dead the morning after administration in spite

of the fact that the two-hour drops in blood-sugar average but 0.054 gram on three tests. In table 5 in one of the cases cited (A-4) there is evidence of action of the product three days after administration. In our earlier work on perfusion we occasionally found evidence of delay in the action of products, so that we feel that delay in response and prolonged action of products are factors in animal variation as well as evidence perhaps of a glucagetic substance occurring with the glucopyretic insulin. Another possible factor is the artificial conditions under which animals must, of necessity, be kept in the laboratory. Loss of appetite is often noted in animals which have been severely affected by products and it seems quite possible that some of the animal variations may be due to relatively poor physical condition. Still another factor which may enter into the production of animal variations is infection. Rather frequently we have seen liver infections (coccidiosis?) post mortem and this condition was found in one of our recent animals (G-3) which showed enormous and unaccountable daily variations in blood-sugar levels.

The comparisons made above between the various groups of table 7 serve to show that animal variation is a large factor in this work and that it must be eliminated before conclusions can be drawn in regard to preparations tested upon normal rabbits.

The Toronto workers point out a variation of +17 per cent to −13 per cent from the average level in one animal on the same day during a period of six hours. This diurnal variation is another important factor in animal variation.

Significance of abnormally low first blood-sugar levels. For the purpose of determining the influence of abnormally low initial blood-sugar levels upon the blood-sugar drops we have collected in table 8 all the tests from our series in which analysis showed that the tests were made upon animals with low initial levels. In each group of tests there is also included one or more tests made upon animals with normal initial levels.

In groups I and II all of the initial levels tend to be low so perhaps no conclusions should be drawn from these.

Group III includes five tests made upon the same preparation. The first three of these were made upon animals with normal initial levels and it will be noted that good check results were obtained. In the last test the initial level was too low and although convulsions were produced the drop was smaller than that of the checks.

In group IV the initial level proved to be 0.013, the animal had convulsions, and was found dead the next day. A drop of only 0.006 gram was produced by the product.

TABLE 8

Effect of abnormally low first blood-sugar levels giving inconstant responses

GROUP NUMBER AND PREPARATION	DATE (1923)	AGE (DAYS)	EQUIV. GM. PAN.	PAN.: WT. RATIO	ANIMAL NUMBER	WEIGHT (KILOS)	1ST BLOOD	2ND BLOOD	DIFF.	CONVULSION TIME	RABBIT UNITS	PER CENT OF 1ST LEVEL REP. BY DROP	REMARKS
I. (1)	5/24	10	3.75	5.00:2K	H-5	2.00	0.081	0.049	−0.032		92	40	75 per cent of dose.
	5/21	7	8.75	5.00:2K	E-1	3.50	0.070	0.015	−0.055	2 hr. 30 min.	157	79	Greater drop. Died 20 min. after conv.
II. (42)	5/26	4	3.00	2.5:2K	F-3	2.40	0.083	0.045	−0.038		217	46	
	6/1	10	2.30	2.5:2K	H-5	1.80	0.066	0.015	−0.051		291	77	Greater drop
III. (41-C)	5/23	2	2.50	2.5:2K	G-6	2.00	0.120	0.071	−0.049		280	41	Good checks—normal bloods
	6/6	16	2.50	2.5:2K	G-1	2.00	0.103	0.044	−0.059		336	57	
	5/25	4	2.50	2.5:2K	G-2	2.00	0.093	0.043	−0.050		284	54	
	5/29	8	3.24	2.5:2K	I-2	2.60	0.089	0.065	−0.024		137	27	Delayed absorption? cf. normal level.
	6/4	14	2.42	2.5:2K	H-4	1.94	0.058	0.016	−0.042	2 hr. 10 min.	240	74	Lesser drop
IV. (3-C)	6/4	11	4.56	5.00:2K	H-6	1.92	0.013	0.007	−0.006	1 hr. 23 min.	17	46	Found dead next a.m.
V. (39)	5/16	7	5.00	5.00:2K	F-5	2.00	0.101	0.030	−0.071	2 hr. 50 min.	202	69	Died over night
	5/15	6	5.00	5.00:2K	D-3	2.00	0.081	0.046	−0.035		100	43	Lesser drop
VI. (40-B)	5/17	2	3.08	2.5:2K	F-3	2.40	0.108	0.017	−0.091	1 hr. 50 min.	521	84	
	5/24	9	2.50	2.5:2K	H-3	2.00	0.081	0.036	−0.045		256	56	
	6/1	17	2.40	2.5:2K	H-4	1.94	0.068	0.019	−0.049		280	72	Lesser drop
VII. (6)	6/13	8	2.85	2.5:2K	J-2	2.28	0.115	0.049	−0.066		377	57	
	6/15	10	2.55	2.5:2K	G-3	2.04	0.077	0.051	−0.026		148	34	Lesser drop
VIII. (5)	6/16	12	3.13	2.5:2K	M-5	2.50	0.115	0.054	−0.061		349	53	
	6/15	11	2.55	2.5:2K	H-2	2.04	0.068	0.036	−0.032		182	47	Lesser drop

In groups V, VI, VII and VIII the animals with low initial blood-sugar levels show a smaller response to the products than do those with normal levels.

In order to determine whether any constant ratio exists between the initial level and the fall caused by products we calculated the percentage of the first level represented by the drop. These results are shown in next to the last column of table 8. An inspection of this column shows that this ratio is not constant and that it does not bear any definite relation to the initial level. In some groups of tests the high ratio is associated with the high initial level, in other groups the opposite condition holds.

In general it would seem that an abnormally low first level would indicate the probability of a smaller response to the product and we can say with certainty that a low initial level produces inconstant results which have the effect of confusing any estimates of the potency of products.

Check tests. In this laboratory we have adopted the method of securing results which check within 0.014 gram before calculating the yield of different products. We feel that until we secure drops which come within this range we are not justified in drawing conclusions in regard to the potency of preparations, owing to the fact that many unavoidable variables enter into animal testing. There is usually not much difficulty in obtaining such check tests when the animals have been allowed sufficient rest between testing periods and particularly when the initial blood-sugar determinations are within the normal range.

It occasionally happens that when a product is tested at the same time upon two different animals one shows a greater response than does the other. In such cases we have adopted the higher figure and have never failed to secure a check for this on further testing. This condition holds only, of course, when normal animals, showing normal initial blood-sugar levels, are used.

A word should be said about the selection of results from rabbit tests. All investigators who have used rabbits for test animals know that they show wide variations in their response to products even after all care has been exercised in selecting and standardizing them. In the absence of a better laboratory method we have selected the maximal response to the smallest dose shown in a series of tests, and have always secured as a check another test which is not more than 14 mg. lower. The criticism may be offered that this does not represent the average response, and this is true. We have been forced to adopt the maximal response (checked as described above) because of the impossibility of carrying out a series of tests on each preparation upon a large enough number

TABLE 9

Duplicate tests made upon different animals of same weight

(Blood-sugar drop absolute)

GROUP NUMBER AND PREPARATION	DATE (1923)	AGE (DAYS)	EQUIV. GM. PAN.	PAN.: WT. RATIO	ANIMAL NUMBER	WEIGHT (KILOS)	1ST BLOOD	2ND BLOOD	DIFF.	CONVULSION TIME	RABBIT UNITS
I. (39)	5/16	7	5.00	5.00:2K	F-5	2.00	0.101	0.031	−0.071	2 hr. 50 min.	202
	5/18	9	5.00	5.00:2K	D-4	2.00	0.085	0.014	−0.071	2 hr. 45 min.	202
II. (40-A)	5/16	1	2.80	2.5:2K	E-6	2.20	0.085	0.025	−0.060	1 hr. 30 min.	343
	5/21	6	2.80	2.5:2K	E-6	2.20	0.092	0.027	−0.065	2 hr. 55 min.	372
III. (41-A)	5/23	2	2.50	2.5:2K	G-4	2.00	0.098	0.072	−0.026		148
	5/25	4	2.50	2.5:2K	H-5	2.00	0.079	0.042	−0.037		212
	5/25	4	2.50	2.5:2K	I-1	2.00	0.073	0.044	−0.029		164
IV. (41-B)	5/23	2	2.50	2.5:2K	G-5	2.00	0.074	0.022	−0.052	2 hr. 50 min.	296
	5/25	4	2.50	2.5:2K	G-1	2.00	0.086	0.037	−0.049		280
V. (41-C)	5/23	2	2.50	2.5:2K	G-6	2.00	0.120	0.071	−0.049		280
	5/25	4	2.50	2.5:2K	G-2	2.00	0.093	0.043	−0.050		284
VI. (41-B-B-P)	6/1	11	2.73	2.5:2K	H-6	2.20	0.090	0.010	−0.080	2 hr.	457
	6/11	21	2.70	2.5:2K	K-3	2.16	0.113	0.026	−0.087	3 hr. 27 min.	497
VII. (4-A)	6/2	2	2.28	2.5:2K	H-5	1.82	0.096	0.027	−0.069	2 hr.	393
	6/12	12	2.28	2.5:2K	H-4	1.82	0.087	0.028	−0.059	2 hr.	337
VIII. (6)	6/13	8	2.85	2.5:2K	J-2	2.28	0.115	0.049	−0.066		377
	6/16	11	2.85	2.5:2K	M-4	2.28	0.101	0.039	−0.062		370

of animals to justify studying averages. We have never seen any objection to studying maximal response to products, since we see numerous possible causes for submaximal responses. Moreover, we have never seen any evidence to support the idea of hypersensitiveness to products and consequently do not feel that the maximal response represents more potency than a product possesses. On the other hand, delay in physiological response unquestionably does occur so that in our two-hour interval we sometimes fail to detect the full response to a product. A practical justification for studying maximal response is found in the clinical use of insulin in that it eliminates the possibility of overdosage if, by this method, preparations receive too high a potency rating.

TABLE 10

Duplicate tests made upon the same animal

(Drop absolute)

DATE (1923)	PREPARATION NUMBER	AGE (DAYS)	EQUIV. GM. PAN.	PAN.: WT. RATIO	ANIMAL NUMBER	WEIGHT (KILOS)	1ST BLOOD	2ND BLOOD	DIFF.	CONVULSION TIME	R.U.
5/16	40-A	1	2.80	2.5:2K	E-6	2.20	0.085	0.025	−0.060	1 hr. 30 min.	343
5/21	40-A	6	2.80	2.5:2K	E-6	2.20	0.092	0.027	−0.065	2 hr. 55 min.	372
5/25	41-C	4	2.50	2.5:2K	G-2	2.00	0.093	0.043	−0.050		284
6/6	41-C	16	2.55	2.5:2K	G-2	2.03	0.103	0.044	−0.059		336

It should be made clear that in all of the tables published in this paper the checked results shown have been selected from a series of tests upon the preparations cited, in conformity to the principles explained above. The actual number of tests from which the selection was made has varied from about four to six. Previous experience with a given type of preparation has been a large factor in guiding us in the selection of the maximal response. In the case of testing entirely unknown preparations we can see no other justifiable method except the determination of the average from a large number of tests.

In table 9 we have brought together duplicate tests made upon animals *of the same weight.* These groups of tests are arranged in order of the age of the preparations. An interval as long as ten days has elapsed in some of the groups—in some of the later tables of check tests a much greater interval has elapsed. In all of these groups of tests it will be noted that the blood-sugar drop is absolute, within the range of variation allowed for colorimetric determination (0.014 gram).

TABLE 11

Duplicate tests made upon different animals

(Drop absolute—independent of 1st level)

GROUP NUMBER AND PREPARATION	DATE (1923)	AGE (DAYS)	EQUIV. GM. PAN.	PAN.: WT. RATIO	ANIMAL NUMBER	WEIGHT (KILOS)	1ST BLOOD	2ND BLOOD	DIFF.	CONVULSION TIME	RABBIT UNITS	PER CENT DROP DIFF. ÷ 1ST BLOOD
I. (2)	5/16	2	6.00	5-2K	E-5	2.40	0.143	0.080	−0.063		186	44
	5/18	4	7.00	5-2K	E-5	2.80	0.095	0.043	−0.052		149	55
II. (3)	5/26	2	6.82	5-2K	F-2	2.72	0.090	0.030	−0.060		172	67
	5/29	5	5.00	5-2K	H-2	2.00	0.089	0.021	−0.068	1 hr. 30 min.	190	76
III. (4-B)	6/9	9	2.85	2.5-2K	J-2	2.28	0.118	0.042	−0.076		435	64
	6/2	2	2.28	2.5-2K	H-6	1.82	0.094	0.014	−0.080	2 hr.	463	85
IV. (41-A-2)	6/8	18	2.50	2.5-2K	G-1	2.00	0.103	0.045	−0.058		331	56
	5/31	10	3.14	2.5-2K	I-4	2.50	0.099	0.028	−0.071	2 hr.	407	72
V. (40-B)	6/11	27	2.85	2.5-2K	K-2	2.28	0.122	0.041	−0.081		463	66
	5/17	2	3.08	2.5-2K	F-3	2.40	0.108	0.017	−0.091	1 hr. 50 min.	521	84
VI. (39-P-3p)	5/23	14	5.00	5-2K	G-3	2.00	0.097	0.050	−0.047		134	49
	5/23	10	7.50	5-2K	F-4	2.96	0.088	0.031	−0.057		163	65
VII. (5)	6/16	12	3.13	2.5-2K	M-5	2.50	0.115	0.054	−0.061		349	53
	6/13	9	2.98	2.5-2K	J-1	2.38	0.097	0.039	−0.058		332	60
VIII. (8)	6/12	1	2.98	2.5-2K	I-4	2.38	0.108	0.039	−0.069		395	64
	6/18	7	3.00	2.5-2K	J-2	2.40	0.098	0.029	−0.069		395	70
IX. (13)	6/19	1	3.10	2.5-2K	M-5	2.48	0.116	0.047	−0.069		395	59
	6/29	11	2.40	2.5-2K	J-6	1.92	0.113	0.054	−0.059		336	52

Table 10 shows the results of tests made upon the *same animal* after a lapse of several days. Here, again, the drop in blood sugar is seen to be absolute.

Assuming that the drop occasioned by a given preparation is an absolute and definite quantity we have followed the common pharmacological practice of administering our products in proportion to the weight of the animals used. Table 11 shows the result of this method. In all of these instances the blood-sugar drop is absolute *when the dosage is proportional to the weight of the animals.*

In the various groups the tests have been arranged so that the animal showing the highest initial level comes first. In all cases the dosage has been made proportional to the weight of the animals. The greatest range is shown in group I. In the first test of this group the initial level was 0.143 gram whereas in the check the initial level is 0.095 (a difference of 0.048 gram) yet the blood-sugar drop is the same in the two animals. In practically all of the groups the initial levels vary yet the drop is absolute *indicating that the fall in blood sugar is independent of the initial blood-sugar level.* In the last column of this table, as in table 8, we have indicated the percentage drop. Inspection of this column shows that this is an absolutely variable quantity which, of course, it must be, if our assumption is correct that the drop is absolute and independent of the initial blood-sugar level.

SUMMARY. 1. The blood-sugar level of normal rabbits averages 0.113 gram with an allowable variation of 0.017 gram above or below this figure.

2. Animals showing abnormally high or abnormally low blood-sugar levels should be rejected for testing purposes.

3. Not more than two tests should be made upon the same rabbit during a week.

4. Convulsions are unreliable criteria for calculating the potency of preparations tested.

5. Dosage should be proportional to the weight of the rabbits used and so regulated that a fall of not more than 0.070 gram is produced.

6. A Rochester "rabbit unit" is an *absolute drop of 0.070 gram* in two hours in a rabbit weighing 2 kilos.

7. The greatest drop produced in a normal rabbit (i.e., one with blood-sugar readings within the normal range) can be taken as a bona fide figure in estimating rabbit units and a check for this figure can, and should, be obtained.

8. Various products may be compared in terms of rabbit units per kilo of pancreas used by means of the formula:

$$R.\ U. = 14.28\ \frac{a}{R}$$

9. Duplicate tests should check within the limits of error of colorimetric readings (0.014 gram).

10. The minimal number of tests made upon a given product should be three—to secure checks and to rule out animal variation.

CONCLUSIONS

If the rabbit is to be used as a test animal for determining the potency of anti-diabetic preparations it is absolutely essential that the animal be normal in every sense of the word, free from the effects of disease, previous injection of products, and with an initial blood-sugar level within the normal range. The animal should not be given doses which will produce a drop in blood-sugar greater than 0.070 gram. The weight should be determined before each injection and the product administered in proportion to the weights of different animals.

Tests which prove to have been made upon animals with low initial blood-sugar levels should be rigidly rejected as valueless.

If the conditions mentioned above are met the blood-sugar drop produced by a given product is absolute—independent of weight and initial blood-sugar level—and duplicate results can, and should always, be obtained within an allowable variation of 0.014 gram.

We wish to express our appreciation of the helpful criticism which Professor J. R. Murlin has given us in this study.

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CONTRIBUTIONS TO OUR KNOWLEDGE OF THE AUTONOMIC CONTROL OF LIVER FUNCTION

I. The Action of Autonomomimetic Drugs, and of Vagal Stimulation upon Fluid and Sugar Output from the Hepatic Veins of the Terrapin

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In two publications from this laboratory dealing with perfusion of the terrapin's liver through the portal vein, it has been shown first by Snyder and Martin (1) that the minute-volume venous outflow *a*, is very susceptible to the C_H of the perfusing fluid (being greatly augmented by increase, greatly retarded by decrease of the hydrogen-ion content), and *b*, that epinephrin in minimal effective concentration, while it increases the augmentor action of perfusing fluids of high C_H, has a contrary action, that is, decreases still further the outflow already retarded by those of low C_H. Second by Snyder, Martin and Levin (2) it was shown that the sugar output per unit of time by the liver followed the minute-volume outflow, whether the changes in minute-volume were effected by changes of C_H or, in addition, by the presence of minimal effective concentration of epinephrin, the epinephrin decreasing or increasing the output of reducing substances, as it did the volume outflow (1), according as to whether the C_H of the perfusing fluid was low or high, that is, somewhat less or more than it is in normal blood taken from the larger blood vessels.

The experience thus gained showed that *a*, the terrapin's liver is an experimental organ of great advantage in any fundamental approach to the problems of liver function; and *b*, that the reverse reaction of minimal epinephrin might be a useful point of departure in attacking the problem of autonomic control.

During the past year accordingly work was undertaken with this objective in view, and the present paper is a report of our results. The methods employed were the same practically as used in (1) and (2), papers which the reader should also consult for additional references.

1. The influence of changes of pressure upon the minute-volume outflow and the output of sugar per minute. In the work already quoted (1), (2) it was pointed out that changes in the pH of the perfusing solution or the addition of epinephrin in minimal concentration seemed to have their effects upon the sugar output from the liver not alone through any specific

effect upon the liver cells but also through their effects upon the caliber of the hepatic blood vessels; if these are constricted by changes of pH or by epinephrin diminishing the outflow, the output of sugar[1] is also decreased; if they are dilated and the outflow increased the sugar output is likewise increased.

It occurred to one of us (Wells) that since this seems to be the case it would be of interest, and becomes really of great importance, to make a special study of the effect of pressure changes upon minute-volume and sugar output from the liver. Here one may increase or decrease the

TABLE 1

DATE OF EXPERIMENT AND OTHER DATA	pH INDEX	PERFUSION NUMBERS	PRESSURE IN CM. WATER COLUMN	MEAN MINUTE-VOLUME OUTFLOW IN CC.	MEAN SUGAR OUTPUT PER MINUTE IN MGM.	PERCENTAL CHANGES IN		
						Pressure	Minute-volume	Sugar output
ii/24/23.—Ringer's plus phosphates and 0.018 per cent dextrose	**6.6**	1–3	6	1.89	0.462			
		5–7	8	2.66	0.817	+33	+40	+77
		8–11	10	6.76	1.60	+25	+154	+96
		12–15	12	9.28	1.81	+20	+32	+13
		16–19	14	16.95	2.42	+16	+82	+33
		20–23	16	19.10	1.61	+15	+12	(−33)
		24	8	2.47	0.665	−50	−86	−58
		25	12	12.50	2.640	+50	+407	+297
iv/7/23.—Ringer's plus phosphate, and dextrose, 0.018 per cent	**6.6**	1–2	12	29.4	3.55			
		3	10	16.65	1.535	−16	−45	−56
		4–5	8	12.5	1.255	−20	−25	−18
		6–7	6	5.12	0.876	−25	−59	−30
		8	4	0.97	0.502	−33	−81	−42
v/5/23.—	**7.65**	1	6.5	0.86	0.218			
		2	8	1.21	0.598	+20	+52	+174
		3	10	1.52	0.99	+25	+25	+65
		4	12	1.47	1.60	+20	−3	+1
		5	16	2.14	1.33	+33	+45	+33
		6–8	20	4.54	1.84	+25	+112	+38

volume flow through the liver in a purely mechanical way independent of chemical means. Will the sugar output still follow the volume flow? Typical results of experiments specially directed to this point are given in table 1.

From these results it appears that the volume outflow and the sugar output from the liver generally increase when the pressure of the per-

[1] The term "sugar" will be used throughout the rest of the paper in the inclusive sense of "reducing substances." In case this is not meant some more specific term such as "dextrose" will be used.

fusing fluid entering the portal vein is increased, and decreases when the pressure is decreased. In this series of experiments it was found again as in the studies of (1) and (2) that changes of pH of the perfusing fluid have a marked effect on the output of fluid and sugar by the hepatic veins.

2. The effect of epinephrin on the portal hepatic vein outflow and sugar elimination. From the results of last year (2) with minimal epinephrin it was inferred by the senior author that this hormone in concentrations higher than minimal would have a constricting action on the portal circulation, whether the C_H be higher or lower than that of mixed blood,—that is, that the preponderating action of epinephrin on the portal-hepatic veins is constricting, and that the remarkable dilating effect gotten by minimal doses was simply another case of the *reversed action* of the drug. The results gotten in the present investigation do not bear out this inference. In presenting the results they are best dealt with in two groups, *a*, those in which perfusing fluids of low pH, and *b*, those in which perfusing fluids of high pH were used.

a. Experiments where perfusing fluids of low pH were used. In the experiment of March 13, 1923, adrenalin-Takamine[2] of various concentrations was used in Ringer's buffered with phosphates to pH = 6.8, and also to which dextrose (Pfanstiehl) to 0.0193 per cent was added. The results are shown in table 2.

Table 3 is taken from the experiment of April 7, 1923, where the dextrose added to the perfusing fluid was 0.018 per cent; and its pH = 6.6.

A similar result was gotten during the experiment of April 21, 1923, (table 4) with buffered Ringer's, pH at 7.35, and dextrose, added to 0.0193 per cent.

The experiment of May 5, 1923, with pH at 6.6, without adrenalin-T, and the perfusion pressure at 20 cm. of a water column, the outflow was 6.66 cc. per minute; but with adrenalin-T, $1:10^8$, added to the perfusing fluid, pressure remaining the same, the outflow increased to 18.6 cc. per minute. No sugar observations were made.

The evidence of the foregoing experiments shows that adrenalin-T in any ordinary concentration when added to perfusing fluids of low pH and sent into the liver through the portal vein at constant head of pres-

[2] Inasmuch as it has been shown that adrenalin-T reduces copper salts in excess of alkali (Abel and Crawford (3)), it was important to know to what extent the doses we used of this substance would affect our results. The protocol on the subject reads as follows:

Blank (without adrenalin-T), titration readings *a*, 19.31 cc., *b*, 19.35 cc., *c*, 19.29 cc.

Blank (with adrenalin-T, $1:10^6$), titration reading, 19.33 cc.

It was concluded that adrenalin-T in the concentrations we used would not introduce an appreciable error in the analyses for reducing substances.

TABLE 2

PERFUSION PRESSURE IN CM. OF A WATER COLUMN	CONCENTRATION OF ADRENALIN-T	MINUTE-VOLUME OUTFLOW IN CC.	MINUTE-SUGAR OUTPUT IN MGM.	PERCENTAL CHANGE IN	
				Minute-volume	Sugar output
10	0	4.35	0.597		
10	$1:10^{8}$	8.1	3.96		
10	$1:10^{6}$	9.15	4.1		
10	$1:10^{5}$	9.55	4.98	+119	+730
8	0	2.77	0.489		
8	$1:10^{9}$	2.94	0.758		
8	$1:10^{8}$	6.61	2.200	+138	+350
6	0	4.26	1.72		
6	$1:10^{4}$	4.4	2.8	+3	+61

TABLE 3

PERFUSING PRESSURE IN CM. OF A WATER COLUMN	CONCENTRATION OF ADRENALIN-T	MINUTE-VOLUME OUTFLOW IN CC.	MITUTE-SUGAR OUTPUT IN MGM.	PERCENTAL CHANGE IN	
				Minute-volume	Sugar output
4	0	0.97	0.502		
4	$2:10^{9}$	2.27	1.94		
4	$1:10^{8}$	2.27	2.2	+134	+340
6	0	5.16	0.817		
6	0	5.08	0.935		
6	$1:10^{8}$	5.08	3.23	+1	+248
8	0	12.5	1.48		
8	0	12.5	1.03		
8	$2:10^{8}$	16.66	5.46	+33	+336

TABLE 4

PERFUSION PRESSURE IN CM. OF WATER	CONCENTRATION OF ADRENALIN-T	MINUTE-VOLUME OUTFLOW IN CC.	MINUTE-SUGAR OUTPUT IN MGM.	PERCENTAL CHANGE IN	
				Minute-volume	Sugar output
10	0	2.975	0.955		
10	0	2.42	0.830		
10	$2:10^{8}$	5.43	6.8	+109	+653
14	0	14.1	5.77		
14	0	11.35	3.9		
14	$2:10^{8}$	36.80	13.2	+189	+173

sure has an augmenting action on the outflow from the hepatic vein and likewise an augmenting action on the output of reducing substances per minute from the liver. Inspection of the columns giving the effects in terms of "percental change," reveals in what striking way under these

conditions the hormone exerts its specific action on the liver cells or some constituent thereof causing them to give up their carbohydrate store to a greater extent than would be expected by the vasodilating action of the H-ions alone, or by the effect of an increase in head of pressure alone. (Compare percental changes in table 1.)

b. Experiments where perfusing fluids of a pH higher than 7.4 were used. In two experiments livers were perfused with fluids of low hydrogen-ion concentration (high pH) with and without adrenalin-T of various con-

TABLE 5

Showing the effect of minimal and higher concentrations of adrenalin-T in perfusing fluids of high pH on minute-volume flow and sugar output from the terrapin's liver[3]

DATE OF EXPERIMENT AND OTHER DATA	PERFUSION PRESSURE IN CM.	ADRENALIN-T CONCENTRATION	MINUTE-VOLUME OUTFLOW IN CC.	SUGAR-OUTPUT IN MGM. PER MINUTE	PERCENTAGE DECREASE (−) OR INCREASE (+)	
					Minute-volume	Sugar output per minute
May 19, 1923.—pH 7.6; dextrose added to perfusing fluid, 0.0198 per cent	2.5*	0	2.2	0.557		
	2.5	0	3.13	1.99		
	2.5	1:10^9	2.13	0.391	−26	−65
	2.5	1:10^9	1.78	0.490		
	3	0	3.82	0.82		
	3	0	4.16	0.69		
	3	1:10^9	4.35	0.205	(+8)	−73
	5	0	24.5	1.82		
	5	1:10^9	20.8	1.0	−15	−45
	7	0	36.7	1.76		
	7	0	20.4	3.44		
	7	2:10^5	27.1	4.10	+34	+19
May 5, 1923.—pH 7.55; dextrose added to perfusing fluid 0.0198 per cent	10	0	1.52	0.98		
	10	1:10^6	3.95	1.55	+135	+63
	10	1:10^6	3.2	1.7		
	20	0	4.54	1.92		
	20	0	4.54	1.77		
	20	0	4.54	1.68		
	20	2:10^5	33.33	3.0	+633	+67
	20	0	6.66	1.45		
	20	5:10^8	20.3	1.5	+204	+3

* This pressure in the protocol is marked "<3". It was not less than 2.5.

[3] The experiment of May 19 was begun with a series of adrenalin-free perfusions where the pressure was discreetly lowered from 12 to 8, to 5 and finally to 3 cm. The sugar-output in those perfusates was smaller than in the corresponding perfusates shown in this table where adrenalin-free and adrenalin-containing perfusions were alternated. The lower sugar output where minimal adrenalin was used therefore may be more apparent than real in this experiment.

centrations. In table 5 are given the results. (These experiments involved a study of the effect of a change of pressure, the results of which will be reported more fully elsewhere. To avoid confusion the reader will bear in mind that at each pressure the outflow was allowed to come to an approximate equilibrium before observations were recorded.)

It will be noted in the first part of the table that the effect of minimal adrenalin in high pH solution is again a depressor of the minute-volume outflow (with one exception), as was observed previously by Snyder, Martin and Levin (2). But the rest of the table, dealing with doses of adrenalin heavier than those authors used, reveals a remarkable result. Here one notes again the marked dilatation and increased output of sugar as were gotten when using fluids of low pH (tables 1 to 4). The dilatation furthermore seems to be greater with greater initial head of pressure. It is remarkable that, although the minute-volume has been augmented from 2 to 7 times over that of the adrenalin-free solutions, yet the minute-output of sugar, while still greater than it is in the adrenalin-free control, nevertheless lags far behind in the increase.

In all our experiments the hepatic artery was left patent but carried no fluid to the liver and this could have added nothing to the venous outflow. There remains however the possibility of a back-flow or shunting of fluid from the portal, by way of the common capillary bed, out through the arterial vessels. In case this actually happened a constricting action of the epinephrin in the arterial vessels alone could result in increased hepatic outflow, without any change of caliber occurring in other vessels. To argue that the increased outflow in this case is due to venous dilatation would obviously be grossly fallacious. The crucial test that an outflow other than through the hepatic veins did not take place was to observe the total volume outflow from the sinus cannula and check this against the total volume inflow. This indeed was the final test, giving the signal to proceed with the experiment.

From the foregoing experiments one may assume that the portal-venous system of the terrapin is supplied with inhibitory or dilator sympathetic fibers, and that the epinephrin, when added to the fluid entering the system in any amounts greater than the minimal effective concentration, augments the minute-volume outflow from the hepatic veins because of its sympathomimetic action upon the myoneural junctions of the muscle fibers in the walls either of the hepatic veins, or of the portal vein and its branches, or of both. It further appears that when the hormone is added in minimal concentration to a perfusing fluid more alkaline than that of arterial blood, then its action is in the reversed direction, that is, it causes even greater constriction than is effected by the increase of OH-ions alone.

3. The effect of pilocarpine and atropin on the portal circulation and the output of sugar by the liver. With the results of epinephrin before us the next question naturally concerns the action of the parasympathomimetic drugs.

The effective concentrations of pilocarpine in perfusing fluids (modified Tyrode's and Ringer's were used) were found to be from 0.024 per cent to 0.048 per cent. But with these doses it was found that pilocarpine could not be removed quickly with the normal perfusing fluid if the livers were exposed to the drug too long. Hence while a number of readings of the controls could be taken in succession (serving thus as checks one upon the other) a less number of readings of the pilocarpine perfusions could be taken. The concentrations of atropin in the perfusing fluids used were 0.008 per cent and 0.01 per cent.

TABLE 6

Showing the effect of pilocarpine upon the portal circulation

DATE OF EXPERIMENT AND OTHER DATA	PERFUSION NUMBER	DRUG USED	OBSERVED MINUTE-VOLUME IN CC.	MEAN VALUES	
				Minute-volume	Percental change
Exper. of iii/3/23.—Ringer's phosphates, pH 7.2; pres. 10 cm. R. temp., 27°C. Observations, time to collect 10 cc. outflow	3	None	2.56	2.73	
	4	None	2.93		
	5	Pilocarpine 2.4:10^6	1.50	1.46	−46
	6	Pilocarpine 2.4:10^6	1.43		
	7	None	2.20		
	8	None	1.82	2.10	+37
	9	None	2.10		
	10	Pilocarpine 2.4:10^6	1.25	1.25	−38
	11	Atropin 1:10^6	2.76		
	12	Atropin 1:10^6	2.93	3.05	+144
	13	Atropin 1:10^6	2.35		
	14	Atropin 1:10^6	2.66		
	15	Pilocarpine 2.4:10^6	1.90	1.73	−43
	16	Pilocarpine 2.4:10^6	1.56		
Exper. of iii/17/23.—Ringer's, pH 7.2; pres. 5 cm. R. temp., 24°C.	1	None	134*		
	2	Pilocarpine 2.4:10^6	93		−30
	3	Atropin 8:10^6	118		+27
	4	None	87		−26
	5	Pilocarpine 2.4:10^6	67		−20

* Min.-vol. for this experiment is given in *drops per minute*.

The method was again the same, the liver by ligation being physiologically isolated from the rest of the animal's body. All *venae cavae* having previously been ligated the perfusion cannula was placed into the portal close to the point where it enters the liver beyond the last of its tributary branches, or if placed below, these intervening tributaries were then tied off. The outflow cannula was tied in the sinus venosus to receive the outflow from the left and right hepatic veins, these entering the sinus directly in the terrapin. Results of experiments with these drugs

are exhibited in tables 6, 7 and 8. In table 6 are given the results of two experiments showing the effect on minute-volume outflow from the hepatic veins, the figures being reckoned from the observed number of cc. or drops from the outflow cannula in a given time. Pilocarpine and atropin were both tested. It will be noted especially by comparing the + and − quantities in the last column, which express the results in terms of percental change, that when pilocarpine is given the minute-

TABLE 7

Showing effect of pilocarpine upon the volume flow and sugar-output per minute from the terrapin's isolated liver

DATE OF EXPERIMENT AND OTHER DATA	PERFUSION NUMBERS	DRUG ADDED TO PERFUSING FLUID	EXTREME OBSERVATIONS		MEAN PERCENTAL CHANGE IN	
			Minute-volume outflow in cc.	Sugar-output in mgm. per min.	Minute-volume	Sugar output
Exper. of Dec. 15, '22.—Ringer's + 0.275 per cent dextrose, pH = 7.15; temp. 25° C., pres., 8 cm.	1,2,3,4	None	19.75 16.75	1.860 0.551		
	5,6,7	Pilocarpine 2.4:10^6	10.18 9.22	0.305 0.823	−46	−57
	8,9,10,11*	None	11.55 14.22	0.696 2.14	+31	+223
	13	Pilocarpine 2.4:10^6	6.70	0.979	−43	−41
	14,15,16,17*	None	10.42 9.30	1.145 0.745	+47	(−3)
	19	Pilocarpine 2.4:10^6	2.30	0.627	−76	−33
	20,21,23,23*	None	2.95 2.30	0.672 0.980	+13	+31

* Nos. 12, 18 and 24 were each the 5th sample of the normal controls and are omitted as supernumeraries.

volume outflow is diminished and that when pilocarpine is withheld from the perfusing fluid the minute-volume outflow increases.

On the other hand atropin following pilocarpine increases the minute-volume output over and above the increase expected by the mere withdrawal of pilocarpine. It was found that atropin, as was to be expected, exerted such long-lasting effects that it could only be used to advantage at the end of an experiment, a procedure that accounts in part for the paucity of our data with that drug.

In tables 7 and 8 are shown results of experiments where both the volume outflow and the sugar output from the hepatic veins were observed. The results shown in table 7 are put in compact form, the two extreme observations for each series of samples taken being given. The effects of changing the perfusing solutions are seen best in the last column in terms of decreased (−) and increased (+) percental change. Here

TABLE 8

Showing effect of pilocarpine upon the portal circulation of, and sugar output from the terrapin's liver

DATE OF EXPERIMENT AND OTHER DATA	PERFUSION NUMBER	DRUG ADDED	MINUTE VOLUME OUTFLOW IN CC.	SUGAR OUTPUT IN MGM. PER MINUTE	MEAN PERCENTAL CHANGE	
					Minute volume	Sugar output
Exper. of 1/20/23.—Tyrode's sol. with 0.1 per cent dextrose; pH = 7.6; pres., 10 cm. temp., 23°C. Min.-Vol. reckoned from time to collect 20 to 25 cc.	1	None	2.16	0.963		
	2	None	1.27	0.740		
	3	Pilocarpine ($4.8:10^6$)	0.93	0.487	−45	−42
	4	None	2.16	1.023	+50	+104
	5	None	1.58	0.970		
	6	Pilocarpine ($4.8:10^6$)	1.42	1.060	−17	−4
	7	Pilocarpine ($4.8:10^6$)	1.66	0.858		
	8	None	1.43	0.785		
	9	Atropin ($8:10^7$)	1.12	0.716	+28	−13
	10	Atropin ($8:10^7$)	2.56	0.643		
	11	None	2.27	0.662	+13	−6
	12	None	1.90	0.603		
Exper. of Dec. 22, '22.—Tyrode's with 0.0876 per cent dextrose; pH 7.45; pres., 8.5 cm. R. temp. 24°–23°C.	7†	None	1.81	0.152		
	8	None	2.64	0.067		
	9	None	2.46	0.23		
	10	Pilocarpine	1.595	0.086	−27	−43
	11	None	1.7	0.296	+66	+229
	12	None	1.71	0.178		
	13	Pilocarpine	1.16	0.126	−31	−47
	14	None	1.36	0.142	+17	+13

† Nos. 1 to 6 gave doubtful results.

the percental change is gotten by a comparison of the means of the extreme observations recorded in the columns to the left.

It is clear in these experiments that perfusing fluids containing pilocarpine and entering the liver through the portal vein pass out in diminished volume per time-unit, the perfusate carrying with it likewise diminished output of sugar per minute. From the experiments where atropin was used (tables 6 and 8) although few in number, it appears that this drug acts as the usual antagonist to pilocarpine, increasing the

hepatic outflow where the latter decreases it. The effect of atropin upon the minute-output of sugar so far as our few results indicate is doubtful. More experiments are needed for a decisive answer.

The results of our experiments with the autonomomimetic drugs on terrapin liver may be summed up briefly by saying that, when the inflow is by the portal vein only, epinephrin increases minute-volume outflow from the hepatic veins and pilocarpine diminishes it; atropin again increases it. Likewise the output of reducing substances per unit of time is increased when epinephrin is given, diminished when pilocarpine is given.

4. On the mechanism of observed drug effects. As to the mechanism of the effect of these drugs upon the sugar output, it appears in the first place that there is an indirect cause. That is, as shown in the experiments where the minute-volume and sugar output from the hepatic veins were changed by mere physical means (by merely changing the head of pressure), so also these drugs in so far as they affect the caliber of blood vessels and thereby affect the minute-volume outflow, just so also do they affect the output of sugar. In the second place so long as the pH is below 7.4 there appears to be a more direct cause. This is shown especially to be the case by our experiments with epinephrin whose results appear in tables 2, 3 and 4. In all other experiments, wherever there is a change in sugar output, while the change follows in the same direction, nowhere does it follow the change of minute-volume in exact or linear proportion. But the difference when epinephrin is given (in low pH) is more marked than in any other experiments, the percental change in sugar output being in every case save one greater than the percental change in minute-volume,—and greater by from two to more than two hundred times. This corroborates the accepted doctrine in a more crucial way on the living organ than hitherto has been done and establishes that doctrine, for our perfusing fluids of pH less than 7.4, beyond all doubt.

On the other hand, a specific action on the liver cells by either pilocarpine or by atropin thus far is not in evidence in our experiments. In the 7 cases where pilocarpine was tried the percental change in the sugar output deviates in three cases more and in four cases less than that of the minute-volume.

5. The effect of electrical stimulation of the vagus trunks. In this series of experiments we isolated the terrapin's liver physiologically again and prepared the vagal trunks in the neck for electrical stimulation. The animals had always been pithed as to the brain and cervical cord, but now the vagi were cut in addition, so that impulses set up at their peripheral stumps in no way could provoke reflexes from brain or cord. The nerves were either stimulated by the bipolar or the unipolar method. The

latter proved the more satisfactory, the indifferent electrode being held snugly to the animal's back with intervening gauze moistened in saline. In case both vagi were to be stimulated simultaneously, the stimulating electrode was bifurcated, the terminal of each bifurcation being put in contact with a nerve. The stimulus used was tetanizing induction shocks (Harvard coil) and the strength of current chosen as adequate was that that was found (in a preliminary test) just necessary to bring the heart to a complete stand-still. Our first experiments gave contradictory results, but the great irregularity in controls indicated faulty technique.

TABLE 9

Experiment of xii/13/'22

Ringer's pH = 7.2; pres., 9 cm.; R. temp., 18–19°C. Time observed to collect samples of 50 cc. each.

ORDER OF OBSERVATION	TREATMENT OF NERVES	MINUTE VOLUME IN CC.	PERCENTAL CHANGE
3	No stimulation	24.8	
4	No stimulation	25.8	+2.4
5	Weak stimulation, left vagus	23.1	−9.1
6	No stimulation	29.4	+27.2
7	No stimulation	26.5	−10.5
8	No stimulation	20.8	
9	No stimulation	20.0	−3.8
10	Stimulation of left vagus (moderate)	10.6	−47.1
11	1st period after stimulation	27.8	+162.0
12	2nd period after stimulation	22.5	−19.0
*			
28	No stimulation	6.2	
29	No stimulation	5.8	−6.4
30	Stimulation of right vagus	4.6	−20.6
31	1st period after stimulation	3.6	−21.7
32	2nd period after stimulation	3.4	−5.5

* Numbers 13 to 27 inclusive contained a technical error and therefore are excluded.

Experience gradually improved the latter and finally we got less doubtful responses to stimulation. These later results are given in tables 9 and 10. They are not very conclusive but still it will be seen that they all point in one direction, namely, that in some way stimuli falling on the peripheral stumps of the cut vagi reach the tissues of the liver, causing probably and primarily a constriction of portal venous circulation and thus a diminished minute-volume outflow from the hepatic veins, and secondarily a decrease in the minute-output of sugar by those veins.

As to direct stimulation of the liver cells by impulses set up in the vagi there appears in these experiments so far only doubtful evidence.

TABLE 10

Showing the effect of electrical stimulation of the peripheral ends of cut vagus nerves upon the minute-volume outflow and sugar output from the terrapin's liver

DATE OF EXPERIMENT AND REMARKS	pH INDEX	TREATMENT OF VAGAL NERVES	PERFUSION ORDER	MINUTE VOLUME IN CC.	MGM. SUGAR PER MINUTE	PERCENTAL GAIN OR LOSS	
						Minute volume	Sugar per minute
Nov. 8, '22.—Ringer's, pressure 8 cm. Both vagi, peripherally by unipolar method; strength, just suffices to arrest heart-beat. 50 cc. samples were taken in most cases, 22 and 23 cc. in nos. 9, 10 and 11	6.7	Before stimulation	1	28.6	3.32		
		Before stimulation	2	28.6	2.52	±0	−24
		During stimulation	3	22.2	2.0	−22	−20
		1st period after stiml.	4	10.4	1.04	−53	−47
		2nd period after stimulation	5	20.0	1.76	+92	+69
		3rd period after stimulation	6	18.2	1.31	−9	−25
	7.9	Before stimulation	7	2.5	0.57		
		Before stimulation	8	2.5	0.76	+39	+33
		During stimulation	9	1.09	0.39	−69	−47
		1st period after stimulation	10	1.04	0.45	−4.5	−15
		2nd period after stimulation	11	1.30	0.56	+25	+24
	6.7	Before stimulation	12	8.42	2.37		
		Before stimulation	13	7.85	1.95	−6.7	−17.7
		During stimulation	14	5.90	1.63	−24.8	−16.4
		1st period after stimulation	15	6.52	1.72	+10.5	+5.5
		2nd period after stimulation	16	5.96	1.64	−8.6	−4.5
		3rd period after stimulation	17	5.32	1.48	−10.7	−9.7
Exper. of Nov. 11, '22 Ringer's; pres., 8 cm. Nos. 1 to 15 incl. were experimentally faulty. Samples taken contained 30 to 50 cc.	7.2	Normal	16	19.6	0.518		
		Normal	17	20.0	0.726	+2.0	+40.1
		Strong stimulation of right vagus	18	20.0	0.506	±0.1	−30.3
		1st period after stimulation	19	14.45	0.101	−27.7	−80.0
		2nd period after stimulation	20	13.8	0.468	−2.2	+363

Of all the tests shown in table 10 the effect of electrical stimulation is greater on the sugar output in two cases while it is greater on the volume

outflow in three other cases. The recovery from stimulation is likewise equivocal, the sugar output being greater in two cases, the volume outflow greater in two other cases. It will be noted the pH was changed and this produced again its usual effect, an effect not recorded in the columns of "percental change."

The failure to demonstrate direct vagal nervous stimulation of liver cells may have been due to the inadequacy of the electrical stimulus used, a point that is to be directly decided by further experimentation. For although the stimulus sufficed to inhibit the heart and set up peristaltic waves in the gut it does not follow that the possible impulses normally exciting liver cells would be of the same character. The last experiment shown in table 10, the one for November 11, 1923, encourages one in this view. Here an unusually strong faradic stimulus was used although the rate of shocks was still the same, about 60 per second. The effect upon sugar output it will be noted is vastly greater than upon volume-flow. Indeed volume-flow undergoes at first no change. There is further a long period of "after discharge," that is the effect of the stimulus is seen to continue during the "first period after stimulation." The "recovery" appears only in the "second period after stimulation" and is likewise marked by little or no change in volume-flow but as much as 363 per cent increase in the sugar output, an increase of the magnitude elicited by decrease of pH so far as the sugar output is concerned, and suggests a condition of asphyxia on account of the vascular constriction.

One point we had in mind at the outset was to show, if possible, the effect of changing the pH of the perfusing fluid upon the character of liver response to nerve stimulation. These preliminary skirmishes with the vagi have served us well in showing that, if pH plays a rôle, it is one that can only be revealed by much more thorough and systematic exploration of not only pH levels but also of the various kinds of electrical stimulation,—using the well-known method of separating excitatory and inhibitory fibers in a common nerve trunk. In this case it may yet be shown that *a reversed response* can be elicited from autonomic nerve stimulation, a response similar to the *reversal* of response to epinephrin.

GENERAL DISCUSSION

1. The effect of epinephrin. It will have been noted already that so far as the glycogenolytic effect is concerned our experiments with epinephrin accord with the findings of others, especially with the work done on mammals. But when we turn to the effect of the hormone upon the vascular reactions of the mammalian liver, our experiments seem to be at variance. For however much explanations of the mechanism of its action may vary, there seems to be a unanimous

conclusion that whenever epinephrin effects the liver vessels at all the action is a constriction of their walls.

This is the view of Burton-Opitz (4), of Macleod and Pearce (5) and of Edmunds (6), all of whom have studied the liver in practically isolated preparations. In our experiments, as has been seen, we have found vasoconstriction only to minimal doses of epinephrin and this only in perfusing fluids whose pH was higher than 7.4. It may have happened that the bloods and solutions perfusing the mammalian livers were always of a pH of 7.4 or greater, but the dose of epinephrin used while oftenest minimal surely was not always so. Will it turn out if the pH and epinephrin concentration are more carefully controlled, that in the mammal as well we shall find vasodilatation to epinephrin in low pH, vasoconstriction only to minimal epinephrin in high pH?

As to the work done by others on cold-blooded vertebrates our results with epinephrin on the terrapin again agree so far as its glycogenolytic action is concerned. But we can not agree with Fröhlich and Pollak (7) when they say that the output of sugar from the liver "as shown on frogs and turtles is independent of the action of adrenalin on the blood-vessels." For as our experiments show, agents causing directly a change in caliber of blood vessels alone also indirectly affect the output of sugar. Adrenalin then must have a double action; one of which is its action on blood vessel caliber and hence on minute-volume flow increasing general irrigation of liver cells thus exposed. Fröhlich and Pollak state that "the portal vessels do not constrict to adrenalin either in weak or strong concentrations (even 1:1000)." So far as dilatation is the negation of constriction, our work supports this statement. These authors however did not control the pH of their perfusing fluids so far as we know. This may account for the difference in our results.

2. The effect of sympathetic nerve stimulation. Experiments on mammals, that have been undertaken to determine precisely the origins, distribution and function of sympathetic nerve fibers in the liver, have had almost a uniform result pointing toward vasoconstriction. It should be pointed out that the earlier experiments involved simultaneous stimulation of nerves supplying the adrenals whose blood was free to find its way to the liver. (Mall, 1890 and 1892; the brothers Cavazanni, 1892–1894; Morat et Dufour, 1894; Bayliss and Starling, 1894.) Indeed Mayer, 1912, and MacLeod and Pearce, 1912, observed no increased hyperglycemia upon puncture of the medulla or upon stimulation of the medulla or upon stimulations of the splanchnics, if the adrenal glands had been removed previously. This view is still held by MacLeod in modified form. (Harvey Lectures, 1914, *Textbook of Physiology and Biochemistry*, 1918.) But nevertheless evidence has been accumulating that direct stimulation of the hepatic nerves

(sympathetics?) with the adrenal secretion controlled, may result in vascular reaction in the liver quite independently of the adrenal glands. (Burton-Opitz, 1910; Trendelenberg and Fleischhauer, 1913; Rossi, 1916.)

In all this work nevertheless nowhere does one get the impression that splanchnic nerve stimulation produces hepatic vein dilatation. However now and then there is a doubt expressed as to any reaction at all of the main trunk of the portal vein (12).

We have not yet undertaken experiments upon mammals, nor have we yet stimulated the sympathetic nerves in the terrapin. Nevertheless the thought can not escape one that if the rôle of normal splanchnic stimulation and of epinephrin is to increase the sugar output of the liver, then simultaneous hepatic vein constriction would involve mechanisms working at cross purposes and represent a maximum of inefficiency. On the other hand it would seem that only under one condition would hepatic vein and artery constriction be an efficient mechanism, namely, when the normal animal is at rest. The tissues and blood would then probably have their highest pH and a minimal content of epinephrin. In the normal, intact terrapin at least under conditions of rest we now would expect to find a decrease in volume-flow and sugar output from the hepatic vein. During excitement and violent muscular exercise, however, when the amount of circulating epinephrin is probably increased and finally when locally the pH is decreased (on account of general abdominal constriction) we would expect to find dilatation of liver vessels especially of the vein and artery, and accordingly increased amounts of blood and sugar poured out from the liver into the general circulation.

3. The effect of pilocarpine and atropin. As to our results with pilocarpine it seems that they are not supported by the observations of others. Cushny (8) says, "The sugar of the blood has been found increased by pilocarpine, and this has been attributed to its acting on the terminations of nerves in the liver which regulate the glycogenic function of the organ." No mention whatever is made of any effect of pilocarpine or atropin on the blood vessels. Brodie and Dixon (9) however found that pilocarpine constricted blood vessels of intestines and limbs and concluded the seat of action to be at the nerve endings. Fröhlich and Pollak (7) on the other hand find that pilocarpine and atropin have no effect whatsoever on the liver.

The reason for this general contradiction of findings may be explained by an inadequacy of the methods employed. In the first place, the liver must be quite isolated physiologically from uncontrolled influences of the rest of the body; in the second place, proper and constant concentrations of the drug, as well as of all the ions in the perfusing fluid, must

be employed; in the third place the head of perfusing pressure must be under constant control, and the volume inflow as well as outflow accurately measured.

The positive result of our experiments showing that pilocarpine decreases the output of the hepatic veins both as to volume of fluid and to weight of sugar per unit of time points to a like effect under vagal nerve stimulation.

4. The effect of vagal nerve stimulatin. In a memoir on *The nervous Regulation of Metabolism*, Stephani (10) reviews at length the work of his associates in this field between the years 1892–1916. The first work on the vagi seems to be that of Vasoin (1903) who found that the glycogen content of vagotomized frogs was greater than that of normal frogs. Much later Roncato (1915) found that stimulation of the vagus on one side of a rabbit, the part of the liver supplied by the other vagus being at the same time ligated, left the part of the liver with vagus intact in possession of a higher percentage of glycogen than the part ligated. Repeating the ligation of the same part of the liver on other rabbits with no nerve stimulation at all gave no such difference in glycogen content between ligated and unligated parts. Rosi (1915) starved rabbits, some with vagi cut below the junction of the inferior laryngeal nerves, and others with vagi intact. After two days the glycogen content of the livers of the vagotomized rabbits was found to be less than it was among the controls. The controls had been subjected to all the procedures, operative and otherwise, that the vagotomized experienced, only the vagi were left intact. Histological studies seemed to point in the same direction (Berti e Roncato, 1910; Berti e Rossi, 1912; and again Rossi, 1915). All these experiments furnished evidence pointing to a direct action of the vagi upon the glycogenic function of the liver, holding in check the glycogenolytic influences exerted upon the organ by splanchnic nerves.

There remains only the possibility that the vagal influence in these cases was not a direct one upon the liver cells or even upon the liver vessels, but an indirect one. For no experiments were untaken to determine to what extent the efferent impulses over the vagal trunks inhibited adrenal secretion or augmented the internal secretions of the pancreas, either one, or both, of which actions we now know would most probably have given the results these authors have described.

In our experiments with the liver in physiological isolation the pancreas and adrenals could have had no effect, and although we made no glycogen analyses of the liver substance yet our sugar analyses of the hepatic outflow (so far as a diminished sugar output may point toward active glycogenesis, or sugar retention) confirm the results of the Italian investigators just reviewed.

It is not without substantial basis therefore to hold the view that some day properly planned and executed experiments upon the mammalian liver will show that parasympathomimetic drugs and vagal nerve stimulation both reduce volume outflow and sugar output, and that sympathomimetic drugs and sympathetic nerve stimulation reverse these processes.

SUMMARY AND CONCLUSIONS

The results of the experiments on the isolated terrapin's liver reported in this paper may be summarized as follows:

1. With changes of perfusion pressure at the entrance of the portal vein the minute-volume and sugar output at the hepatic veins are both changed in the same direction as the pressure, so long as the pressure varies between 3 and 20 cm. of a water column. The extent of the change in output of fluid and sugar is not always linear with the change of pressure, nor is the extent of change in amount of fluid and of sugar commensurate one with the other, so far as one can judge from the data here presented.

2. The effect of adding epinephrin to the perfusion fluid in concentrations, varying from 1 part epinephrin: 10^3 parts fluid, to 1 part epinephrin: 10^4 parts fluid, all produce increased minute-volume outflow and still greater increased output of sugar per minute so long as the pH of the perfusing fluid is less than pH = 7.4. This effect on minute-volume outflow is also the same with perfusing solutions whose pH is a little more than 7.4 provided the concentration of the epinephrin is greater than the minimal effective dose. When the minimal effective concentration of epinephrin ($1:10^9$) is present in the perfusing fluid and the fluid is more alkaline than that of normal arterial blood then the effect of the drug is reversed, the minute output of fluid being decreased beyond the output obtaining when the same perfusing fluid without the hormone is used. It is clear now that *the glycogenolytic function of epinephrin is probably confined in the normal animal to blood of low pH, and to blood of high pH only when epinephrin is present in greater dosage than the effective minimal. If the pH is slightly greater than 7.4 and only a minimal effective dose of epinephrin is present then the minute-outflow from the liver is decreased more than it would be without the drug*, that is, decreased more than what it would be by mere increase of OH-ions alone.

As already stated, the effect of epinephrin upon the output of sugar usually is much greater than it is upon the volume-outflow; this is true whether the effect is augmentatory or inhibitory (reversed effect with minimal epinephrin in high pH). This fact corroborates the view of a specific action of epinephrin upon the liver cells or upon a part of their contents, and is in accord with experiments *in vitro* (Langfeldt).

3. Pilocarpine added to the perfusing fluids of various pH elicits only a decreased output of both fluid and sugar by the hepatic veins. A few experiments with atropin indicate that it also here antagonizes the action of pilocarpine, and furthermore that it probably of itself has a direct effect increasing hepatic vein outflow and sugar output.

4. The effects of these socalled autonomomimetic drugs suggest corresponding effects when nervous stimuli through the autononomic nerves reach the vascular walls and liver cells. Experiments stimulating faradically the peripheral ends of the vagi thus far only reveal a vasoconstrictor action, that is, a diminished minute-volume outflow and a diminished output of sugar at the hepatic veins. This result confirms the action of pilocarpine, indicating that the terminations of the vagal fibers have a vasoconstrictor effect on the walls of the portal system or of the hepatic veins or of both. The fact that to ordinary faradic stimulation the output of sugar is not affected in a greater degree than what the vessel walls are, points to the change of sugar output in this case being probably dependent only upon the volume outflow.

In one experiment where very strong faradic stimulation was employed, however, there is a much greater diminution of sugar output than there is of volume-outflow. This indicates that further experiments may still show that certain forms of vagal nerve stimulation have a specific inhibitory action upon the liver cells, one that may even point to a positive glycogenic influence.

5. It is purposed to extend these studies *a*, to a further exploration of vagal nerve action; and *b*, to an investigation of the direct action of sympathetic nerves of the liver under various pH levels.

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SOME CONDITIONS AFFECTING CATALYTIC ACTION OF BLOOD[1]

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The catalytic activities of blood and tissues have been investigated by numerous workers and many papers have been published on the subject, but no positive conclusion has as yet been reached as to its significance in the body, and little seems to be known about its properties.

Burge refers to it as an enzyme, and since his observations tend to show that wherever oxidations are increased, catalase is also increased, he concludes that it is an enzyme particularly concerned with oxidation.

Burge's results and conclusions have been criticised as being inaccurate and unreliable. R. J. Seymour (1) in his article reports no relation between the heart rate and catalase content, but criticises his own peculiar results as being probably due to error of method rather than to any true difference in catalase content.

The difficulty experienced by most observers in obtaining constant results has led F. C. Becht (2) to conclude that "the catalase cannot be particularly important" and that a study of catalase cannot possibly explain the mysteries of the process of oxidation. He found the blood from the same individual in duplicate and triplicate determinations to give results frequently differing by 50 per cent and even 100 per cent.

Reimann and Becker (3) came to a similar conclusion as a result of their experiments on the effect of anesthesia upon catalase, since they found that anesthesia produced an increase in 35 per cent and a decrease in 65 per cent of the cases.

Morgulis (4) criticises the method usually used in measuring catalase activity because of the large amounts of hydrogen peroxide used, which amounts he, as well as some of the older investigators, found to produce depressing effects. Because its activity is affected by so many factors, he makes a final conclusion that "little credence can therefore be given to results of catalase experiments unless very large differences are demonstrated."

[1] Part of the expense incurred in this research was covered by a grant of the Research Committee of the University of Kansas.

Ruth Okey (5) more recently abandons the method usually employed because of the complicated apparatus needed to prevent supersaturation of the reaction mixture with the oxygen liberated, and concludes that determinations must be made under much more carefully controlled conditions than in the past, if they are to be of value for diagnostic purposes, or to indicate the trend of oxidative processes of the animal organism.

She used a method employed by some of the European investigators in which the catalase activity was measured by determining the amount of undecomposed hydrogen peroxide left in a solution after allowing the catalase to act for a given time on a measured amount of hydrogen peroxide. The reaction was then brought to an end by the addition of a measured amount of 10 per cent sulphuric acid and the undecomposed peroxide was estimated by titration with standard potassium permanganate.

The data given in this paper resulted from an attempt to lessen the sources of error accompanying the determination of catalase in blood, with the hope of obtaining more constant results. We feel justified in presenting our findings, since they corroborate many of the results of previous workers, and point out some of the conditions which we found to be responsible for variable results.

A. Discussion and data on methods of experimentation. The chief causes for irregular results were found to be, first, variations in amount of blood due to error in accurately measuring the small portion used for each determination; second, change of temperature during the interval of the experiment; third, the reaction of the hydrogen peroxide used; fourth, the length of time in which the catalase was allowed to act; fifth, the interaction of the above factors. We have investigated each of these factors and submit our methods and results under the following headings:

a. Measuring. The first cause of error was reduced to a minimum by using Ostwald's $\frac{1}{2}$ cc. pipette. When as little as $\frac{1}{2}$ cc. of blood is used even a difference of a small fraction of a drop will cause an appreciable variation in the oxygen reading.

When the Ostwald pipette was used, and other conditions kept constant, the results came within the experimental error. In some of the later experiments where smaller amounts of blood were used, a $\frac{1}{10}$ cc. graduated pipette was employed, or one to nine dilutions were made.

b. Hydrogen peroxide used. F. and G. hydrogen peroxide purchased in eight pound bottles from the Faxon & Gallapher Drug Company, Kansas City, Missouri, was used for all these experiments. The same stock solution of hydrogen peroxide was used for a series of experiments on individual samples of blood, and these were performed in as short an

interval of time as possible, since it was found that when a new stock supply of hydrogen peroxide was opened and used, there was an average increase of 52½ per cent in the amount of oxygen liberated during a ten minute interval.

Stehle (6), Bodansky (7) and Burge (8) also observed that different samples of the same make of hydrogen peroxide gave varying results with the same blood.

This we found by titration to be due to a difference in the acidity between the freshly opened bottle and one from which we had used for some time. The new sample of hydrogen peroxide required 3.94 cc. n/50 sodium hydroxide to neutralize and liberated 130 per cent more oxygen than the old sample which required 6.13 cc. of n/50 for sodium hydroxide neutralization.

The difference in the acidity of the various samples of hydrogen peroxide was also observed by Bodansky (7), Norgaard (9) and others.

No attempt was made to keep the reaction of the different samples of hydrogen peroxide constant, since the same sample was used for a given series of experiments and conclusions were drawn from each series separately.

c. Apparatus. The method used by Burge (10) was employed with certain modifications. The measured amount of blood was placed in a small clean dish. This was carefully lowered into a wide mouth bottle containing 50 cc. hydrogen peroxide. This bottle was fitted with a rubber stop which in turn was connected by rubber and glass tubing with an inverted burette previously filled with a measured amount of water. After all connections were made, the liberated oxygen given off when the blood and hydrogen peroxide were allowed to mix for a given time, was collected in the burette tube under water and the reading again taken. As any change in the temperature of the hydrogen peroxide mixture during the observed interval was found to affect the amount of oxygen given off the temperature of this mixture was kept constant during the timed interval, by placing it in a water bath heated or cooled to the required temperature, and, in every case, the barometric pressure and the room temperature were taken, and the oxygen volume was reduced to standard conditions. The burette tubes were kept in a deep cylinder of water at room temperature and the readings always taken at the water level of the cylinder.

Most investigators have used a constant temperature, Seymour (1) observing that to use the same temperature was most important. Ruth Okey's (5) work on temperature effects resulted in similar conclusions.

d. Shaking. As far as we are aware, all investigators have seemed to consider it important that the hydrogen peroxide and blood be shaken

at a constant and definite rate per minute. We did not find the constant shaking to be an important factor. In fact, in nine out of eleven averages, slightly more oxygen was liberated when shaken intermittently than when shaken with a definite number of shakes per minute. The averages for our data show that when the samples were shaken continuously at about 120 double shakes per minute, either by hand or by machine, the average amount of oxygen liberated was 16.09 cc., while when shaken less frequently the average was 16.96 cc. In only two cases were the amounts greater with continuous shaking, and then the average increase was only 0.55 cc. in ten minutes.

In cases where the catalase is very active during the first five or ten minutes, more frequent and vigorous shaking was found to be necessary.

e. Experimental data. Defibrinated blood was always used and over 600 determinations were made. Blood was taken from at least 37 different dogs, 9 human beings, several cats and rabbits, and 1 sheep.

B. TEMPERATURE AS A FACTOR IN THE ACTION OF CATALASE: *a. Temperature of the hydrogen peroxide blood mixture.* As has been said, it was observed that a change in the temperature of the hydrogen peroxide blood mixture during the interval of the experiment resulted in the liberation of variable amounts of oxygen. This observation led to a series of experiments to ascertain to what extent temperature affects the activity of catalase. A summary of these results was published by us (11) in a preliminary report. Figure 1 shows curves plotted from the average amount of oxygen liberated by the activity of the catalase from the blood of 6 dogs, 2 rabbits and 9 human beings. In these determinations the catalase activity was tested with temperatures ranging from −2°C. to 40°C. Curve *c* represents an average from blood samples of five human beings taken in November, 1920, and curve *d* that of samples from four human beings taken in January, 1921.

In every case where dog's blood was used, there was a marked decrease in the amount of oxygen liberated as the temperature was raised from −5°C. or −2°C. to as high as 44°C. The average amount of oxygen liberated in 10 minutes by the catalase from 6 dogs at −2°C. was 46.97 cc.; at 10°C. it was 33.49 cc.; at 22°C. it was 25.09 cc.; at 30°C., 19.35 cc.; and at 40°C., 15.64 cc. were liberated.

Very high temperatures were not tried as the higher temperatures produce some decomposition of the hydrogen peroxide. At −2°C. and below the hydrogen peroxide blood mixture was usually partly frozen, but even while thus frozen, the oxygen was liberated at a rapid rate. The amount of oxygen released by the action of ½ cc. of dog's blood at −2°C. in ten minutes time was from 57 per cent to 120 per cent greater than that released at 22°C. The catalytic action of

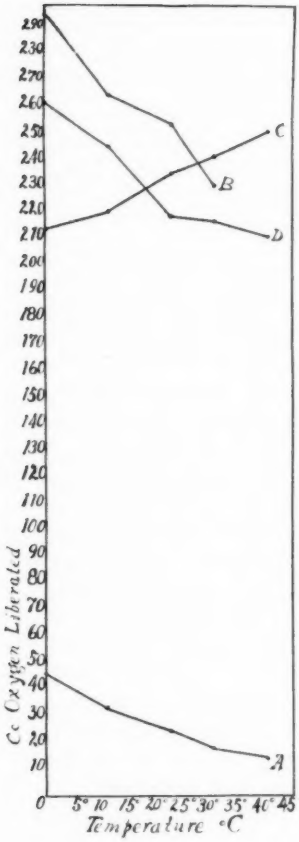

Fig. 1

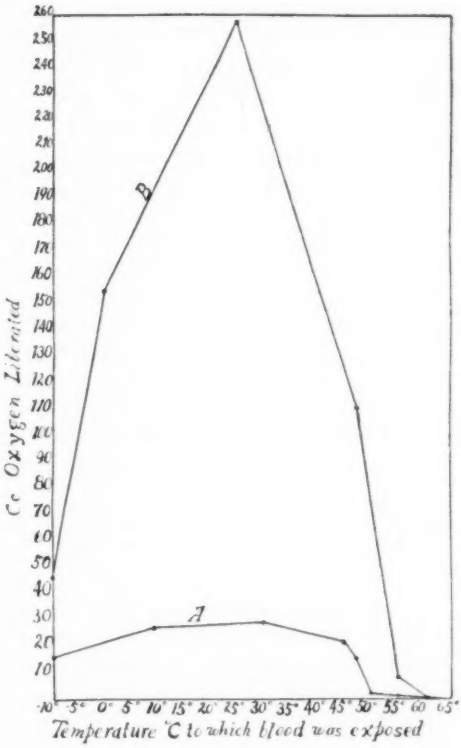

Fig. 2

Fig. 1. Effect of temperature on the action of catalase

Curve *A* averaged results of catalase activity from 6 dogs.
Curve *B* averaged results of catalase activity from 2 rabbits.
Curve *C*, averages from 5 human beings taken November, 1920.
Curve *D*, averages from 4 human beings taken January, 1921.

Fig. 2. The action of blood catalase as determined at 22°C. following its exposure to different temperatures for 20 minutes.

Curve *A*, averaged results from 4 dogs.
Curve *B*, effect of temperature exposures on rabbit's blood catalase.

dog's blood at temperatures between −2°C. and 44°C. is practically inversely proportional to the temperature of the blood-peroxide mixture.

The observations made on the rabbit's blood showed less increase in oxygen liberated at the lower temperature than did the dog's blood, the catalase activity at −2°C. being only 13.7 per cent to 17 per cent greater than at 22°C. The average amount of oxygen liberated during the same interval of time by the blood catalase from the two rabbits when tested at −2°C. was 295.15 cc.; at 10°C., 265.1 cc.; at 22°C., 255.15 cc.; and at 30°C., 230.65 cc.

Temperature effects have since been tried on blood drawn from one cat with results similar to those obtained on the dog's blood.

The results obtained on the samples of human blood are less constant. The data reported by us (11) on the five individuals showed a slight decrease in catalytic action at the lower temperatures (−2°C. to −5°C.), and a slight increase as the temperature was raised up to 40°C. These results were obtained from blood drawn in the month of November. In the following January, samples were drawn from four different individuals with somewhat different results from those above noted. In these four cases the amount of oxygen liberated at 0°C. was more than at 40°C. and in every case there was a decrease in activity with the increase in temperature of the blood-peroxide mixture. The averages for the November readings were as follows: −2°C., 214.81 cc.; 10°C., 220.17 cc.; 22°C., 237.16 cc.; 30°C., 241.81 cc.; and 40°C., 251.01 cc. Those for January were: 0°C., 262.1 cc.; 10°C., 247.12 cc.; 22°C., 218.87 cc.; 30°C., 217.1 cc.; and 40°C., 211.9 cc.

More data on human blood will be needed before any conclusions can be drawn, however, as no attempt was made at this time to control the reaction of the peroxide and, as will be discussed later, that is a most important factor when working with the different temperatures.

b. The action of blood catalase tested at 22°C. and at 0°C. following its exposure to different temperatures for twenty minutes. To determine whether catalase is destroyed when exposed to high and to low temperatures for twenty minutes, a series of tests was made. It was found that blood drawn from six different dogs when tested at 22°C. had lost practically all its catalytic activity following exposure to temperatures between 47°C. and 50°C., decreasing very rapidly when raised above 45°C.

When the blood was exposed to temperatures between 0°C. and 45°C. very little loss in catalytic activity occurred. When exposed to temperatures from −10°C. to −14°C. the blood lost from 30 per cent to 50 per cent of its catalytic action. It was also found that blood kept below 0°C. for twenty minutes, if then tested with the peroxide mixture at 0°C. no more oxygen was liberated than if tested at 22°C. The con-

tinued low temperature had destroyed the property of the catalase, that of increased activity at a low temperature.

Rabbit's blood was subjected to the various temperatures for fifteen-minute periods with results similar to those obtained with the dog's blood. Figure 2, *a*, shows a curve plotted from the averaged results obtained from the several samples of the dog's blood catalase, following its twenty-minute exposures to the various temperatures. Curve *b* shows the effect of temperature exposure on the rabbit's blood catalase.

Our results do not agree with those of Euler (12), as quoted by Ruth Okey (5), who, she says, found increased activation when his saline suspensions were heated to temperatures of 55 to 57°C. She reports some apparent activation at the higher temperatures during the first few minutes but states that Euler's results were very imperfectly confirmed. Their results may be accounted for on the fact that they heated diluted mixtures of blood. In every case we heated the whole blood.

Our results indicate that catalase is rapidly destroyed at a temperature between 47° to 50°C. and is only slightly affected by freezing temperatures, thus resembling enzymes. It differs from other enzymes, however, in that it is very active, and under certain conditions more active at lower temperatures than at body temperature. This, as will be shown later, is true only when the peroxide is acid in reaction.

c. The effect of temperature on the amount of oxygen liberated in the different hydrogen peroxide mixtures. A series of experiments was performed which will be described later in this paper, in which the catalytic action of blood was tested with 50 cc. of hydrogen peroxide and compared with different mixtures of hydrogen peroxide.

Apparatus were arranged so that four tests could be made simultaneously under exactly similar conditions, except that of the blood-peroxide mixtures which were as follows: Solution I, 50 cc. hydrogen peroxide; solution II, 50 cc. hydrogen peroxide made isotonic (0.9 per cent) by the addition of sodium chloride; solution III, 50 cc. hydrogen peroxide made isotonic (0.9 per cent) with sodium chloride and alkaline by adding ½ cc. normal sodium carbonate; and solution IV, 50 cc. hydrogen peroxide made alkaline by adding ½ cc. sodium carbonate without the sodium chloride.

These four test mixtures were tried with blood from seven dogs and one sheep, and determinations were made for each at 0°, 10°, 22° and 37°C. The four mixtures were tested at the same temperature simultaneously. Figure 3 shows curve plotted from the averaged results of the dogs' blood. The sheep's blood traced practically the same curve.

In the case of the blood from the seven dogs tested with solution I (untreated hydrogen peroxide) in every instance the greatest catalytic activity took place at 0°C. and the least action at 37°C. These results

were the same as our previous tests on temperature had shown; but in solutions II, III and IV, where sodium chloride or alkali or both had been added to the peroxide, in every case but one, there was an increase in the activity of the catalase as the temperature was increased. The exceptional case was ½ cc. dog's blood treated with solution II. In this instance ½ cc. non-diluted blood was used. In all other tests $\frac{1}{10}$ cc. non-diluted blood, or 1 cc. of a 1–9 dilution was used. Where the larger portion of blood had been employed, the activity was highest at the lower temperatures as had always been the case when non-treated peroxide was used.

However, in solutions III and IV where sodium carbonate had been added, the amount of oxygen collected was always greater even at 0°C. than that of the non-treated hydrogen peroxide at zero temperature; but the increase of the treated over the non-treated hydrogen peroxide was less at 0°C. than at the higher temperatures because solution I showed the highest catalytic action at 0°C. while solutions II, III and IV gave their highest readings at 37°C. In case of solution II, where sodium chloride alone was added, and tested at 0°C., there was less activity over the non-treated hydrogen peroxide, except in the one case above noted where the larger amount of catalase had been used. These results alone show how important it is to control the temperature along with the reaction of the peroxide, since the effect of the addition of sodium chloride and alkali depends on the temperature at which it is tested.

Most of the later investigators have realized that the temperature and the reaction of the hydrogen peroxide were both important factors, but, so far as we know, they have not shown the interdependence of the two factors.

The above results indicate that when greater catalytic action is obtained at lower temperatures it can be accounted for by the fact that the catalase is not destroyed or inhibited as rapidly at lower as at higher temperatures. When the catalase is protected by sodium chloride in solution or by alkalinity, the catalytic action is more nearly proportional to the temperature although it does not follow Van't Hoff's law. Under the most ideal conditions we could produce, the catalytic action was surprisingly high at the lower temperatures.

C. HYDROGEN PEROXIDE MIXTURES AND DILUTIONS: *a. The effect of adding sodium chloride and sodium carbonate to hydrogen peroxide and testing at 22°C.* Hydrogen peroxide mixtures were prepared as described above in solutions I, II, III and IV, and tested from ten to fifteen minutes at 22°C. Blood from 14 dogs, 1 sheep and 1 rabbit was tested in the usual way. In every case when the hydrogen peroxide was made isotonic with sodium chloride and tested at 22°C., the cata-

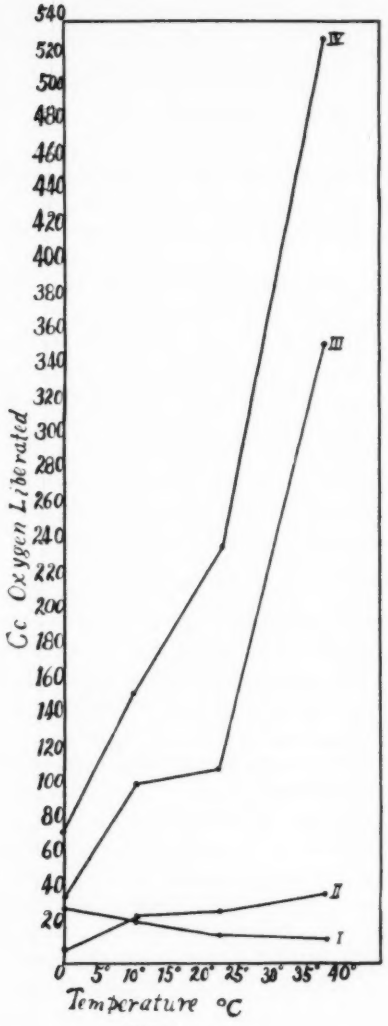

Fig. 3

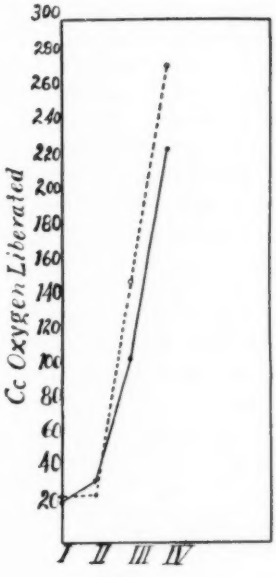

Fig. 4

Fig. 3. The effect of temperature on the amount of oxygen liberated from dog's blood catalase tested in different hydrogen peroxide mixtures.

Curve *I*, solution I, untreated H_2O_2.

Curve *II*, solution II, H_2O_2 made isotonic 0.9 per cent with NaCl.

Curve *III*, solution III, H_2O_2 made isotonic 0.9 per cent with NaCl and alkaline by adding ½ cc. normal Na_2CO_3.

Curve *IV*, solution IV, H_2O_2 made alkaline by adding ½ cc. normal Na_2CO_3 without the NaCl.

Fig. 4. Effect of adding NaCl and Na_2CO_3 to H_2O_2 and testing at 22°C.

I, solution I, untreated H_2O_2.

II, solution II, H_2O_2 made isotonic 0.9 per cent with NaCl.

III, solution III, H_2O_2 made isotonic 0.9 per cent with NaCl and alkaline by adding ½ cc. normal Na_2CO_3.

IV, solution IV, H_2O_2 made alkaline by adding ½ cc. normal Na_2CO_3 without the NaCl.

•———• Averaged results of blood from 7 dogs.

○----○ Result of blood from 1 sheep.

lytic action was increased, the average increase from 7 typical results of dog's blood being 54.1 per cent. The increase in catalytic activity for the sheep was 8 per cent and for the rabbit it was increased 150.8 per cent.

When ½ cc. normal sodium carbonate was added (solution III) with the sodium chloride, there was an average increase for the dog's catalase over the isotonic peroxide alone, of 196 per cent. When the sodium chloride was omitted and the peroxide made alkaline, the increase was still greater, it being 532 per cent. Thus the addition of the sodium chloride alone increased the action over that of the non-treated or acid hydrogen peroxide; but when the peroxide was first made alkaline (solutions III and IV) the presence of the sodium chloride always inhibited the action in the above series by 52.12 per cent. Figure 4 shows a curve plotted from these results. Ruth Okey (5) reports a slight slowing down of the rate of action when the peroxide dilution (1–9) she used contained sodium chloride in a concentration of about 0.89 per cent as compared to the same amount of peroxide diluted 1–9 with distilled water. She used neutral hydrogen peroxide, however, in 1–9 dilution and diluted blood. This slowing we always found to be true in the case of the alkaline hydrogen peroxide. We did not add sodium chloride to neutral peroxide; but when it was added to acid peroxide, the activity was always increased except as previously noted, when tests were made at low temperatures.

These results show that the catalytic activity at 22°C. is always increased by the addition of sodium chloride to acid hydrogen peroxide in a concentration of 0.9 per cent, but when added to the alkaline hydrogen peroxide it inhibits the activity of the catalase. It would, therefore, appear that sodium chloride protects the catalase from the retarding influence of the acid. If, however, the catalase is acting in the more ideal alkaline medium, the sodium chloride inhibits its activity.

c. Effect of diluting neutral hydrogen peroxide with distilled water. Our data on the effect of diluting the hydrogen peroxide are too incomplete to be of much value. In one series of experiments the peroxide was made neutral, then diluted up to 50 cc. with distilled water in proportions 1/4, 1/2, 3/4 and 4/4 hydrogen peroxide, and to each 1.2 cc. neutral sodium carbonate was added. In the tests, there was a decided drop in the oxygen collected as the peroxide became more dilute.

Evans (13) has shown that for each concentration of enzyme there is an optimum concentration of hydrogen peroxide. This factor was not controlled in our experiments.

Morgulis (4) states that with a constant quantity of catalase, as the concentration of the hydrogen peroxide increases, the reaction proceeds more slowly. His conclusions are not very clear but tend to show a

direct proportionality between the amount of catalase and the amount of oxygen liberated from a given quantity of hydrogen peroxide which, he says, holds true only within certain limits. He has proven, however, that the depressing effect is not due to incidental impurities in the hydrogen peroxide or to the destruction of the catalase by oxidation. He furnishes evidence which indicates that catalase reacts with a definite quantity of hydrogen peroxide, and that it is used up in the reaction. He used slightly acid peroxide for all his determinations which he admits is depressing, and therefore it would seem to render his conclusions of questionable reliability.

D. Effect of different dilutions and amounts of blood and age of the samples: When the hydrogen peroxide was made slightly alkaline the catalytic action was always so much greater that we found it necessary to use smaller amounts of blood. This made possible more accurate readings of the amount of oxygen liberated in short intervals of time. A series of tests was made with 0.1 cc. of non-diluted blood and these were compared to blood dilutions of 1–9 with distilled water or 0.9 per cent sodium chloride solutions. The results showed such slight differences as to make dilution or the kind of dilution negligible. The average of 13 tests diluted with water liberated 18.04 cc. oxygen and the 13 tests diluted with 0.9 per cent sodium chloride solution gave an average of 18.44 cc. of oxygen. No change in catalytic action occurred when the diluted blood was allowed to stand as much as two days. This agrees with what other workers have reported.

We did not establish a proportionality between the concentration of the catalase and that of the hydrogen peroxide; but for our experimental conditions where 50 cc. of hydrogen peroxide were used, the increase in the amount of oxygen liberated was always in direct proportion to the amount of blood added which holds true at least when determinations are made for half-hour periods. In a typical example in one series where rabbit's blood was used and the activity measured for a half hour at 22°C., 0.01 cc. of blood liberated 53.7 cc. of oxygen; 0.02 cc. of blood gave 111 cc. of oxygen, or practically double the amount obtained with 0.01 cc. For 0.03 cc. of blood the reading was 169.5 cc. of oxygen; and for 0.04 cc. it was 222.7 cc. of oxygen.

E. Changes in the alkalinity of the hydrogen peroxide tested at 22°C. A great many tests were made to ascertain the optimum hydrogen ion concentration that would give the greatest catalytic activity. The hydrogen ion concentration of the hydrogen peroxide mixtures was tested without blood and after the catalase had completed its action with practically the same results. The reaction having taken place at the different temperatures did not change the hydrogen ion readings.

We found the optimum hydrogen ion concentration for the catalase activity to lie between pH 8.7 and pH 9, or an average of 9 determinations to be 8.9 for fifteen and twenty-minute periods. This hydrogen ion concentration is somewhat higher than that reported by Ruth Okey (5), Morgulis (4) or Bodansky (7). Sorensen (14), as quoted by Bodansky (7), finds the optimum hydrogen ion concentration for catalase to depend upon the duration of the reaction. Bodansky (7) under his experimental conditions finds the optimum to be about pH 7.5 for ten minutes, and about pH 8 for five-minute periods. All investigators seem to agree that acid and too great an alkalinity inhibit or destroy the catalase action. We also found this to be true.

F. TIME REACTIONS AND FACTORS THAT INFLUENCE IT: Our results indicate that with optimum conditions the catalytic activity is continued for many hours. The relation between the concentration of the hydrogen peroxide and the amount of the catalase is a condition that we have not controlled, which might be a factor in producing optimum results.

We have used different portions of blood, however, but always the same amount of hydrogen peroxide, except in the tests noted already where water dilutions were made. In the first series of experiments, $\frac{1}{2}$ cc. of defibrinated blood was used; but in the later tests where alkali was added to the hydrogen peroxide, the amount of oxygen liberated was so great that it was necessary to use smaller amounts of blood. While the catalytic activity was different for each animal used, the catalase for the same animal was found to decompose hydrogen peroxide in direct proportion to the amount of blood used.

When blood was mixed with untreated hydrogen peroxide and tested at temperatures above 22°C., the activity had practically ceased within the first four or five minutes. When zero temperatures were employed, the total amount of oxygen liberated in ten or fifteen minutes' time was much greater than at 22°C., or above, and the activity was also greatly prolonged. The length of time for which activity could be maintained was not determined, but in several cases the action continued for more than one hour.

The greatest volume of oxygen was collected and the longest periods of activity were observed, when the optimum amount of alkali was added to the hydrogen peroxide and the tests made at 22°C. or up to about 37°C. Higher temperatures were not tried because of the unstability of the alkaline peroxide if determinations are made much above 22°C. and this decomposition of the hydrogen peroxide at the higher temperatures increases with the alkalinity. Careful tests were made to determine the amount of this decomposition. With optimum

alkalinity for catalase activity, it was found that an average of 14 cc. oxygen per each ten-minute period might be liberated by the action of the alkali alone if tested at 37°C. If determinations were made at 22°C. the average decomposition was only 1.3 cc. oxygen per ten minutes, and at lower temperatures practically no decomposition occurred. When very small amounts of blood are used, as was necessary in case of rabbit's catalase, and tested at 37°C., they must be carefully checked with a control experiment to determine the amount of activity not actually due to the catalase. In such an experiment it was found that practically all the catalase action was completed during the first ten minutes. This was not true when tests were made at 22°C. or below. In such a series of experiments, it was found that the greatest amount of oxygen collected in an hour due to catalytic action was obtained at temperatures at or below 22°C. This seems to indicate that the catalase is actually used up or destroyed more rapidly at the higher temperature. When larger amounts of blood were used, however, the hydrogen peroxide decomposition due to catalase is entirely out of proportion to that due to the alkali, and the greatest activity in alkaline hydrogen peroxide really occurs at the higher temperatures, as was shown in our earlier experiments. Morgulis (4) states that his experiments were made in a medium slightly on the acid side (pH 6.7 to 6.9) in order to guard against spontaneous decomposition of the hydrogen peroxide, which occurs most readily at and above pH 7.0. Our control experiments in which we tested the amount of decomposition of the hydrogen peroxide by the alkali without the blood indicate that optimum alkalinity may be used without introducing an error into the results if the determinations are made at or below 22°C.

So many factors are found which influence the activity of the catalase in a given sample of blood that it does not seem that we can conclude with Morgulis (4) that there is necessarily a great excess of catalase if the reaction is so vigorous as to be completed in ten or fifteen minutes, or that the catalase is used up because its activity has ceased. We have recorded many instances where the catalytic action continued for three hours or more. The action is usually complete and the catalase apparently destroyed in less than ten minutes when the peroxide is acid in reaction and the temperature is 22°C. or above. If the catalase is exposed to peroxide slightly alkaline in reaction, its activity will continue for a long time if the temperature is not too high. If the temperature is raised to 37°C., nearly all action of the catalase is completed within ten minutes. The decomposition of peroxide which takes place after that time we found to be about the same as would occur without addition of catalase.

DISCUSSION. The results obtained in the present investigation help to confirm the conclusions drawn by others, that conditions for catalase determinations must be very carefully controlled if they are to be of any practical value. Too much credence should not be given to conclusions drawn from catalase determinations made in short periods of time unless results under various conditions are carefully compared.

Most of the later investigators refer to it as an enzyme and much of the data we have presented indicates that many of its properties resemble those of the enzymes found in the body. One outstanding difference, however, is its remarkable activity at the lower temperatures.

We found it to be an agent rapidly destroyed if exposed for fifteen to twenty minutes to temperatures above 47°C., and only slightly affected by an exposure to zero temperatures. In this respect it does resemble ordinary enzymes.

Our results show it to be an agent quickly made inactive but not used up or destroyed in a slightly acid hydrogen peroxide and capable of becoming active when the medium is made alkaline.

Its remarkable activity when allowed to act on acid peroxide at the lower temperatures, indicates that at these temperatures it is protected by the cold and thus acts more vigorously and for a longer time than it does if exposed to the higher temperatures. It may be that the acid effect of the acid peroxide as an inhibitor to the catalytic activity is increased as the temperature is increased, thus causing the reaction to reach an equilibrium sooner at the higher temperatures and allowing the greatest liberation of oxygen at the lower temperatures.

The reversal in temperature effects when isotonic sodium chloride or alkali or both are added, indicates an added protection or activation and now causes the activity to be increased as the temperature is raised. There is an optimum hydrogen ion concentration, however, and when the hydrogen ion concentration is too high, the catalytic action is inhibited or destroyed.

While the sodium chloride seems to protect or activate the catalase acting in an acid medium, except at the lower temperatures, when the peroxide is made alkaline inhibitory effects were always produced by the sodium chloride.

In making catalase determination, therefore, certain factors must be carefully controlled: sodium chloride effects, the temperature and the alkalinity, but the most potent of all seems to be the alkalinity. The reactions of the hydrogen peroxide should always be checked and the determination made at or near the optimum hydrogen ion concentration and at a constant temperature not above 22°C.

SUMMARY

1. A careful study has been made of the methods usually employed in catalase determinations and some of the causes for variable results have been reported.

2. When commercial (acid) hydrogen peroxide is decomposed by dog, cat, or rabbit blood catalase at temperatures between −2°C. and 44°C., the oxygen liberated is practically inversely proportional to the temperature of the blood-peroxide mixtures. Similar results were obtained in a few cases with human blood.

3. *a.* Non-diluted defibrinated blood subjected to temperatures between 47°C. and 50°C. for twenty minutes, when tested at 22°C., is found to have lost practically all its catalytic activity.

b. Twenty minute exposures to temperatures between 0°C. and 45°C. produce very little, if any, change in its activity.

c. Blood exposed for twenty minutes to temperatures between −10°C. and −14°C. loses from 30 to 50 per cent of its activity and also its property of increased activity when tested with acid hydrogen peroxide at the lower temperatures.

4. *a.* When hydrogen peroxide is made isotonic 0.9 per cent by adding sodium chloride, the catalytic action is usually inhibited when tested at the lower temperatures, and increased at the higher temperatures, as compared to the action on hydrogen peroxide when no salt has been added.

b. When hydrogen peroxide is made alkaline the activity varies directly with the temperature if measured for the first ten minutes.

c. If the alkaline hydrogen peroxide is also made isotonic by adding sodium chloride, at every temperature the action is inhibited, but it is still greater at all temperatures than the isotonic acid hydrogen peroxide and follows the same law of increased activity as the temperature is increased.

5. We did not find the catalytic action to increase when neutral hydrogen proxide was diluted with distilled water.

6. Defibrinated non-diluted blood was compared with 1–9 water dilutions and 1–9 physiological salt dilutions with practically the same results.

7. The average optimum hydrogen ion concentration for fifteen and twenty-minute periods was found to be pH 8.9. Acid and too great alkalinity inhibit or destroy the catalytic activity.

8. The time required for a reaction to reach its equilibrium was found to depend upon the temperature of the blood-peroxide mixture and the hydrogen ion concentration of the hydrogen peroxide. With optimum alkalinity and temperature of 22°C. or better, catalase continues to act for three hours or more.

9. If the action of catalase is completed in ten minutes or less the medium is responsible for some destruction of catalase. Determinations of catalase should therefore be made under conditions which permit its action to continue as long as possible. We have found that at optimum hydrogen ion concentration and temperature of 22°C. very constant and reliable determinations of blood catalase can be made if the action is allowed to continue at least one-half hour.

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EXPERIMENTAL ABLATION OF THE SUPERIOR CERVICAL SYMPATHETIC GANGLIA OF THE RABBIT

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Meltzer, experimenting with rabbits, established a hypothesis that the superior cervical ganglia of this animal are essential for the maintenance of life (1). He leads one to believe that the requisite factor lies not within its anatomical connections, but is due to an internal secretion. Montgomery (2) published the results of his series of extirpations in rats, cats and rabbits while the investigation reported herein was in progress. He maintains that continuance of life is not only possible but to be expected, following bilateral superior cervical ganglicctomy. Complete removal was verified in each instance by the paradoxical pupil dilatation by adrenalin and by a careful histological study of the neck tissue, proximal to the ganglia.

This is more or less substantiated by the fact that the removal of the ganglia of the human is not attended by a high mortality. This excision, known as Jonesco's operation, has been performed for glaucoma, tic douloureux, epilepsy and exophthalmic goiter.

An additional interesting phenomenon that we have observed is the hypopyrexia that follows the removal of the ganglia. The rectal temperature of the rabbit may gradually drop from 38.5°C. before to 33.8°C., 34.4°C., 31°C., three days after the operation. The low temperature at this late period excludes anesthetic effects. Bernard originally thought that the cervical sympathetic system contained calorific fibers, but later these were found to be vasoconstricting in nature. The question which concerns us at present is whether the lowered temperature is due to the increased heat dissipation accompanying the vasodilatation or whether there is in addition a thyroid factor of temporarily lowered metabolism.

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THE INFLUENCE OF VARIOUS DIURETICS ON THE CONCENTRATION OF THE BLOOD

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Within the last few years, the regulation of the blood volume has engrossed the attention of many investigators. Despite the diverse methods of approaching the solution of this problem, the interpreted data are nevertheless quite valuable.

It is an axiom of physiology that the composition of the blood is constant. Normally the water content of the blood constitutes one of the body's constants.

To us blood concentration may be effected in one of two different ways. In the first place one may imagine that a fluid, nearly simple water, or a dilute solution, in composition, leaves the blood vessels in a response to the proper stimulus, resulting in a more concentrated blood, a dehydration, a desiccation as it were. Or again by a different type of mechanism, or perhaps as a result of a different form of stimulus, fluid of the nature of dilute plasma rather than of a salt solution passes through the vessels leaving behind a blood concentrated above normal.

In a consideration of the development of pulmonary edema in gas poisoning, one of us (F. P. U.) wrote the following:

> The lethal war gases are all substances eminently irritant to living tissues and it must be accepted that the irritation produced by a gas is the initial step in the development of edema. In response to the first irritative stimulus tissue fluid finds its way to the injured area in an apparent attempt toward repair or alleviation of the injury. It is conceivable that if damage to the tissue is only slight such a procedure would result in the passage to the damaged area of only a small quantity of tissue fluid. According to this view the degree of response with respect to the local deposition of tissue fluid would be in direct ratio to the extent of injury. On the other hand, it is equally plausible to assume that this reaction may reach a breaking point at a certain degree of stimulation whereby the whole mechanism governing the exudation of tissue fluid is thrown out of control, so that the response to the stimulation becomes overwhelming (5).

The pulmonary edema of influenza produces a similar blood concentration picture (4). Observations in recent cases of extensive superficial burns (4 a) lead one to believe that the quantity of fluid lost in

this way during the first few hours after the injury may be very large, in fact sufficiently great to account for the rapid blood concentration which occurs.

Other abnormal conditions which produce a marked concentration of the blood are Asiatic cholera; cholera infantum (2); shock, whether arising spontaneously from trauma or toxemia, or evoked experimentally by peptone (6), or histamine; toxemia from Vaughan's crude soluble poison (7); dysentery; acute arsenic poisoning and peritonitis.

If one analyzes these pathological states it must be quite evident that the one underlying factor common to all, with the exception of shock, is that an extensive inflammatory reaction is prominent, acting usually upon a more or less restricted area, as the respiratory tract, the skin, the alimentary canal, the peritoneum, etc. All are areas plentifully supplied with capillaries.

In order to understand the nature of the mechanism producing the blood concentration a word as to the character of the fluid lost from the blood is essential. From observations on gas poisoning it becomes apparent that this fluid partakes of the nature of plasma, diluted plasma as it were, containing somewhat less protein than plasma, but otherwise of practically the same composition. The fact that significant quantities of proteins are present and indeed the blood proteins, particularly fibrinogen, leads to the view that the irritant factor has changed the character of the capillary wall. In ultimate analysis therefore one may conclude that the direct cause of blood concentration, in the pathological states under discussion, is due to a changed permeability of the capillary wall.

In most of these instances actual loss of fluid to the body has occurred sufficient in amount to account for the concentration of the blood. With shock, however, where no loss of fluid to the body takes place the explanation of the mechanism is not so obvious. Under shock conditions increased capillary permeability may also explain the mechanism involved since the fluid leaving the vessels is plasma (Bayliss, Dale). The toxic substance responsible for the condition acts more or less specifically upon the capillaries resulting in a pouring of fluid into the tissue spaces of the body, thus provoking an exceedingly rapid and marked blood concentration.

From our experience and conception of the mechanism involved in the loss of plasma from the blood a change in capillary permeability is essential—in the loss of salt solution no such alteration is necessary. Blood concentration induced by loss of water and presumably salts only, results in a concentration by a process of dehydration or real desiccation. This is what occurs when sufficient fluid is not introduced as by experimental water deprivation of animals (8), (9), or clinically

in the dehydration of infants. Again intense secretion induced by pilocarpine (3) or purgation by the saline cathartics (1) will cause a rapid blood concentration.

Blood concentration of this type is obviously of an entirely different nature than that induced by an inflammatory reaction. This becomes quite apparent when attempts are made to restore the blood to its normal concentration. In the former case administration of fluid is all that is essential (10). With the concentration induced by an inflammatory reaction administration of fluid alone although helpful is not strikingly successful. Why? Because in the latter instance the capillary wall has been modified so that it is no longer capable to the normal extent of retaining fluid. In other words, in the one type of blood concentration the capillary wall is involved, in the other it is not, at least to an extent capable of measurement.

If the above mentioned factors are capable of producing a concentration of the blood by facilitating the egress of fluid from the vessel walls, deductive reasoning would conclude that diuretics might not be without a similar influence. Both mechanisms in the production of egress from the capillaries may be instrumental in the causation of diuresis. The saline diuretics apparently act by salt action: other diuretics, as the caffeine allies have been stated to be stimulators of kidney secretion, acting by increasing the permeability of the glomerular capsule. The urine in the latter instance resembles plasma more closely than does the urine of saline diuresis. The question might be asked: "If there is any analogy between capillary permeability and diuresis, why do not the host of pathological conditions, characterized by an increased capillary permeability, produce a ready diuresis?" Naturally, the markedly lowered blood pressure accompanying these conditions is sufficient in itself to answer this question. As was previously stated, this permeability may be regional depending on the location of the irritative stimulus to the capillary wall. The diuresis or increased kidney permeability ascribed to caffeine is compatible with this hypothesis, as it is generally agreed that caffeine acts somewhat specifically upon the glomerular capsule.

In attacking this problem several questions present themselves. Will diuresis measurably modify blood volume? Is diuresis possible beyond a certain degree of blood concentration? Does blood dilution insure diuresis in the intact kidney? If blood changes do occur following the use of various diuretics, could they be attributed to the extrarenal action of the drug, e.g., on capillary permeability?

It is common knowledge that nephritis, especially with fluid retention, is accompanied by anemia. This anemia may be only more or less apparent, being simulated by a dilution of the blood (11). Hence

if renal inability is etiologically responsible for the hydremic plethora, can we not inversely presuppose that an effective diuresis would concentrate the blood?

EXPERIMENTAL. *A. The index of blood concentration.* The present blood volume methods are unfortunately inaccurate. From the experimental viewpoint, the relative rather than the absolute blood volume is of more pertinent interest. When the procedure is properly controlled, the interpreted data may be of significant import both diagnostic and prognostic, even though the premises upon which the method is based are fallacious.

In the present problem the hemoglobin values are assumed to be a working criteria of the relative blood concentration. Recent argument (12) has been advanced that the blood cells and plasma are not uniformly mixed in all parts of the circulatory system, the arterioles, for example, presenting an excess of plasma over red cells (13). The principle from which the latter conclusion is drawn is that the arterioles possess a marginal "still space" in which a considerable amount of plasma may be stored. Apparently, there is not only a normal quantitative local variation in red blood cells and consequently hemoglobin (14), but also an irregular distribution and a variability of the number of red cells in circulation, may be evident in pathological conditions (15) of experimental (16) or clinical origin. Other investigators (17) conclude that hemoglobin values are good indices of relative blood volume, insomuch as the hemoglobin content follows the similar and parallel curve of blood total solids determination (4).

Hemoglobin was determined after the method of Cohen and Smith (18), being expressed in percentage of the initial rather than the absolute value. The blood was always obtained from freely flowing sources, usually clean-cut incisions in the marginal ear veins of the dog. We frankly confess our inability to overcome an experimental error of 5 per cent, due to slight discrepancies in blood flow, pipette measurements, similar small variations in the quantity of N/10 HCl diluent, and the personal factor in colorimetric readings.

B. Methods of procedure. Full-grown female dogs (multiparae) were employed because of the greater ease of catheterization. Water deprivation was enforced for the twenty-four hours preceding the experiment, in order to eliminate or control water diuresis. Hemoglobin samples were taken every fifteen minutes throughout the course of the experiment. Catheterization was practiced hourly, except in two instances when thirty-minute intervals were employed, and in one instance when a residual catheter was retained in the bladder. None of the laboratory animals developed cystitis under the carefully controlled management. The urine volume was measured. After the

experiment had progressed for one or two hours, sufficient to establish control values, the drug was exhibited via syringe and needle into the jugular vein. Asceptic precautions were always preserved. Anesthesia was not induced except in two instances when the kidneys were ligated.

Cognizance was taken of the fact that, to insure diuresis, a certain amount of water must be present to be eliminated. Accordingly each drug was given with and without water, which fluid was given in the proportion of 50 cc. per kilogram of body weight. The method of administration was by stomach tube, at a time immediately preceding the intravenous injection of the drug. The complete experiment consumed from five to eight hours.

The following schema of investigation was utilized:

1. The normal variations of hemoglobin content and urine output in dogs, with and without fluid intake.

2. The influence of intravenous injections of hypertonic saline solutions upon hemoglobin content and urine output in dogs, with and without fluid intake. Sodium acetate in 40 per cent aqueous solution was given in doses corresponding to 1 gram per kilogram of body weight.

3. The influence of intravenous injections of hypertonic urea solutions upon hemoglobin values and urine output under similar conditions of water intake, as employed above. Urea in 40 per cent aqueous solution was given in doses corresponding to 1 gram per kilogram of body weight.

4. The influence of intravenous injections of pituitary extract upon hemoglobin per cent and urine output in dogs, under conditions similar to the above. The dosage employed was 2 cc. of pituitrin (Parke, Davis).

5. The influence of various caffeine derivatives (intravenously injected) upon hemoglobin per cent and urine output, under the same conditions of fluid intake as stated previously.

a. Caffeine—10 cc. of 2 per cent citrated caffeine solution was the dosage employed (19).

b. Theobromine—Sodium salicylate (diuretin). Dose per kilogram of body weight was $\frac{1}{2}$ cc. of 10 per cent aqueous solution.

c. Theophyllin—Sodium acetate (Theocin). Dose per kilogram of body weight was $\frac{1}{2}$ cc. of 4 per cent aqueous solution.

6. The influence of strophanthin (intravenously injected) upon hemoglobin per cent and urine output, under similar conditions of water intake. Dose, per kilogram of body weight was $\frac{1}{12}$ to $\frac{1}{18}$ cc. of $\frac{1}{10}$ per cent aqueous solution.[1]

THE NORMAL VARIATIONS OF HEMOGLOBIN CONTENT AND URINE OUTPUT IN DOGS, WITH AND WITHOUT FLUID INTAKE. *Without fluid.* The normal hourly urine volume of dogs under these control conditions is between 2 and 6 cc. The hemoglobin percentage fluctuates slightly, but within the limits of normal variation.

[1] Additional methods of procedure, carried out in certain instances to prove a related point, will be discussed in detail in their respective locations.

The charts shown in this paper, are merely illustrative examples of a series of similar experiments.

With fluid. Water is the diuretic par excellence, but unfortunately it is not universally applicable, e.g., in the relief of the edema of cardiac decompensation. Water is absorbed in unappreciable quantities from the stomach; however, it passes rapidly into the duodenum (30 minutes), whence it quickly enters the body proper. Although absorption occurs throughout the intestine, small and large, the major portion of the fluid intake is absorbed in the duodenum and upper jejunum (Moritz). The various factors controlling intestinal absorption (Goldschmidt) may be dismissed from consideration, with the exception of osmosis, which no doubt plays the chief rôle in the mechanism of the absorption of anisotonic and hypoisotonic solutions.

The water enters the blood as a dilute saline solution (Cushny), the salt content being added in the passage. Cow (20) maintains that, in addition to the salt acquisition, the ingested fluid contains a specific substance from the gastro-intestinal mucous membrane, which sub-

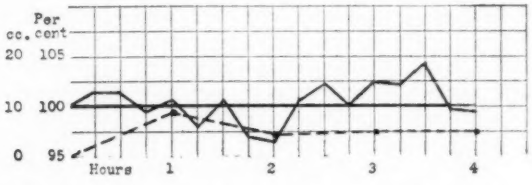

Chart 1. Dog 1. Weight, 7.6 kgm. Control. Without fluid. ——— Hemoglobin. ---------- Urine volume (hourly).

stance is probably acquired in the transit of the water and brings about diuresis by a hormonal-like action, stimulating internal secretions.

The introduction (per os) of large volumes of water into normal individuals alters the blood concentration little or not at all. It is the nature of the organism to maintain its circulating fluid, the blood, in constant proportions (21). Priestley has been able to demonstrate a very slight decrease in the electrical conductivity of the blood, subsequent to the ingestion of huge quantities of water (22). He believes that a minute decrease in the salt concentration of the blood is responsible for the diminution in electrical conductivity, and that the kidney is diuretically sensitive to changes in the concentration of the blood. Such delicate variations are not made manifest by estimations of the hemoglobin content, since we have never succeeded in detecting a dilution of dog's blood, following ingestion of 50 cc. of water per kilogram of body weight.

Diuresis begins immediately after absorption of the water, and continues as long as this excess fluid reaches the blood, even though

no blood dilution is apparent. This early observation may be made, that hydremia is not an essential requisite for the augmentation of urine output. The quantity of water here administered insures diuresis, since it has been established that polyuria appears quickly following the oral ingestion of water, in excess of from 10 to 15 cc. per kilogram of body weight (23). In our experiments the elimination of this excess fluid starts 60 minutes after the time of intake, the height of the excreting curve occurring about 120 to 150 minutes subsequent to the administration. This is a longer period than that found by other investigators; Cow, 70 minutes, Mattill, 90 minutes.

Oncometer experiments show an increase in kidney volume during diuresis (20). According to Cushny (24), when the absorbed fluid is carried to the kidneys "it is filtered off in a form slightly more dilute

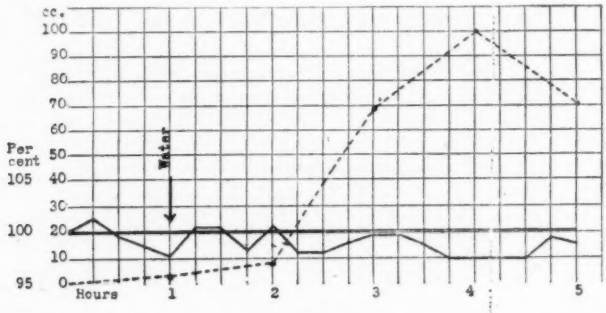

Chart 2. Dog. 1. Weight, 6.3 kgm. Control. Water intake: 315 cc. of water by stomach tube (50 cc. per kgm.). ——— Hemoglobin. ---------- Urine output (hourly).

than the optimal fluid," a contention corroborated by Burian (25) in his experiments tending to show that the glomeruli secrete a very dilute solution after large amounts of water. It would appear, in this early discussion, that the kidney is the regulating factor in the maintenance of a blood concentration level, but this has not been proven. The ability of the tissues, in their rôle as fluid reservoirs, has yet to be considered.

THE INFLUENCE OF INTRAVENOUS INJECTIONS OF HYPERTONIC SALINE SOLUTIONS UPON HEMOGLOBIN CONTENT AND URINE OUTPUT IN DOGS. Sodium acetate was chosen as the most typical saline diuretic, because of the potential possibilities of action it possesses. It may act by increasing the volume of blood through the kidney, directly stimulating the secreting cells, lessening absorption from the tubules and liberating colloidal water.

Sodium acetate is oxidized in the body to sodium bicarbonate, carbon dioxide and water. $NaC_2H_3O_2 + 4\,O = NaHCO_3 + CO_2 + H_2O$. The carbon dioxide combines with the basic elements of the blood and is excreted as carbonates, rendering the urine alkaline. The H-ion concentration of the blood is slightly lessened. It is well to take note of the fact that the capacity of the human organism to oxidize acetic acid is limited, and some of the injected salt will appear in the urine unmodified.

Just as acidosis decreases the urinary flow by increasing the water-holding capacity of the body colloids (26), so does the alkalinity of the acetates produce diuresis by a dehydrating action on colloids, thereby releasing free water. Renal secretion is dependent on free available water (27). The colligative properties of sodium acetate and its oxidized derivatives still further enhance its diuretic properties, insomuch as the injection of its hypertonic solution (40 per cent in these experiments) causes an ingress of tissue fluids into the capillaries, in response to the osmotic attraction exerted there. This further is responsible for a dilution of the blood colloids, synergistically facilitating the increase in urine output (28), which is, in this sense, partly a "dilution diuresis." The theory has been promulgated by some authors that even intravenous saline possesses diuretic properties, not only because of its free state (29) but also by virtue of its dehydrating power (30).

When 40 per cent sodium acetate solutions are rapidly introduced intravenously, a hydremic plethora invariably results for reasons mentioned above. This is true of all hyperisotonic salt solutions (31). We have previously shown that hydremia does not occur with water intake, as the hypothesis is hereby established that a dilution of the blood, although not essential, may be concomitant with diuresis. The power of the blood to resume its original concentration is nothing short of remarkable. In the majority of our dogs the maximal dilution occurred within a half-hour after the intravenous injection; the concentration being restored to normal limits within one and a half to two hours. After the intravenous injection of a quantity of physiological saline solution equal to the calculated blood volume of the animal, it has been found that complete restoration of the original blood volume takes place within 30 minutes (17), (32). The prolonged dilution in our animals may be attributed to the intense concentration of the injected fluid, although this would tend to be equilibrated by the rapid exodus of some of the injected molecules, into the tissue fluids. When the molecular concentration of the blood becomes normal, the ensuing behavior is similar to introduction of isotonic solutions, and restitution to normal blood volume speedily occurs.

The muscles supply most of the water entering the blood vessels, and they also take up some of the salt (33).

On observing chart 3, it will be noticed that the height of urine excretion occurs at a time when the blood concentration has been restored to within normal limits. This may be explained by the rapid loss into the tissues, of the diluting fluid, when isotonicity is established. Sollmann's resumé of the facts cannot be improved upon: "The tissues act the part of elastic reservoirs, quickly relieving the blood of the disturbing element, to again give up the abnormal substances as rapidly as they can be removed by the more slowly acting excretory mechanisms." The capacity of the tissues to absorb fluid is relatively enormous, e.g., the tissue of rabbits may absorb fluid approximately four times the normal blood volume of the animal (17). Engels (34) in studying the water depots of the body following intravenous injections of salt solution, found that the muscles, skin and kidneys take

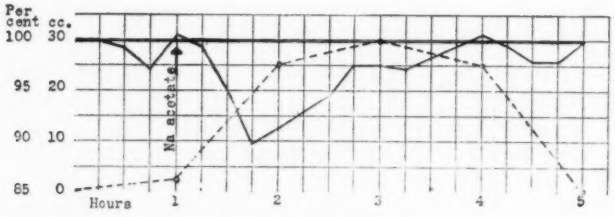

Chart 3. Dog. 1. Weight, 6.4 kgm.; 16 cc. of 40 per cent sodium acetate given intravenously (1 gm. per kgm. of body weight). ——— Hemoglobin. ---------- Urine output (hourly).

up the greater amount of water, but that all the tissues except bone become more watery. The chief place of storage of the excess salt (colloidal adsorption?) is the skin and subcutaneous tissues (35); the storage and release of which salt is quite slow (due to the relatively small proportion of the blood normally passing through the skin and adjacent connective tissue (36)). This may explain the prolongation of diuresis in our experiments beyond the time when the blood is restored to normal concentration.

The modifying influence of sodium acetate upon the concentration of the blood evidently does not necessarily occur through the medium of diuresis, but rather by its action on fluid and salt interchange and capillary permeability. The diuresis is the incidental result, not the cause. Indeed, the activity of the kidneys is not essential to the regulation of blood volume, following intravenous saline injections (37), since it occurs even after ligation of these organs (17). From a

broad viewpoint one may confer upon the tissues (extra-renal and extra-vascular) the emergency and temporary function of blood volume regulation, the kidney serving as the relief by finally removing the excess of fluid and salt.

The mechanism of saline diuresis appears at first glance to be quite simple but, delving into the multiplicity of theories submitted in various texts, one is apt to become confused. A short summary of the factors involved is hereby given for evaluation:

1. Increased filtration pressure (difference between the pressure in Bowman's capsule and in the glomerular capillaries). This may be produced by

a. Increasing the total quantity of blood. Fischer and Knowlton maintain that the volume of blood is not essential, since replacement of the colloid by salt solution, results in a marked diuresis. We prefer to limit this statement to the increase of free water in the blood.

b. A relative or absolute decrease of blood colloids, thereby reducing osmotic resistance to filtration (Cushny). This lessens hemic viscidity as well as lessening friction in the capillaries.

c. Hyperisotonic solutions, such as 40 per cent Na acetate, increase filtration pressure by osmotic shrinkage and dehydration of the renal cells.

2. Specific stimulation of renal cells (Magnus). Sollmann suggests a possible depression of the reabsorbing cells. Current opinion dictates the acceptance of renal cell activity during saline diuresis, as identical to a dead membrane, since Sollmann's reproduction of saline diuresis in dead kidneys.

3. Increased volume of kidney. This may be either the result of the specific stimulation mentioned above, or it may be due to the hydremic plethora induced by the hypertonic Na acetate solution. Our oncometric experiments definitely showed an increased kidney volume, the general blood pressure remaining unaltered.

4. Rapid flow of urine through the tubules (flooding) prevents reabsorption (Cushny), just as cathartic salts act by preventing absorption from the intestine, using McGuigan's simile.

With fluid intake. The simultaneous introduction of water (per os) further complicates the problem that confronts us, but is valuable for accentuating the phenomena which occur. The ingress of fluid from the tissue spaces into the blood vessels does not occur without the expenditure of quite an amount of work. The influx of the excess free water more easily satiates the avidity of the hypertonic blood, so that hydremia results more easily and to a much greater degree than with the Na acetate injection alone (see chart 4). This is indeed, a very interesting fact, as we remember that the introduction of an equivalent amount of water alone does not modify blood concentration. The increased blood dilution in this experiment over the previous one leads us to infer that water and intravenous Na acetate are mutually synergistic. This supposition is moreover confirmed by the fact that not only is there a greater diuresis with fluid intake and Na

acetate injection as would naturally be expected, but the maximum peak of urine output occurs three hours after exhibition of the drug and the water, whereas in the instances with water intake alone the greatest urine excretion was four hours after injection. The reason for this may be that the increased osmotic attraction of the hypertonic blood facilitates and hastens the absorption of water from the intestines. We are aware of the fact that the rate of diuresis is more or less dependent on the concentration of the intravenously injected solution, whereas the degree of diuresis depends on the quantity of saline solute and water introduced (31).

Our data evidently do not agree with those obtained by Baird and Haldane (36), who declare that "the excretion of salts and water

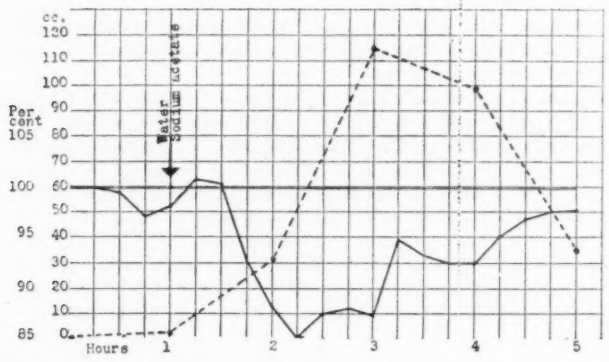

Chart 4. Dog 3. Weight, 5.8 kgm.; 290 cc. of distilled water by stomach tube (50 cc. per kgm. of body weight); 12 cc. of 40 per cent sodium acetate solution intravenously (1 gm. per kgm.). ———— Hemoglobin. ---------- Urine volume (by hours).

after the injection of hypertonic solutions, followed by copious water drinking, shows that the diuresis produced is independent within wide limits of the amount of water ingested."

THE INFLUENCE OF INTRAVENOUS INJECTIONS OF HYPERTONIC UREA SOLUTIONS UPON HEMOGLOBIN CONTENT AND URINE OUTPUT IN DOGS. Urea has been empirically designated as a "saline diuretic." Although relatively non-toxic, since enormous quantities may be injected with impunity, yet it has not merited therapeutic usage. Urea has been suggested as the means of an accurate kidney function test, because its quantitative estimation in the urine (being excreted almost in toto) establishes a measure of the kidney's ability to excrete a normal urinary constituent.

The diuretic action of urea is somewhat similar to saline diuresis, but the mechanism is not comparable in its entirety. Urea, like free molecular sugar, is an organic crystalloid, a non-electrolyte. Incapable of ionization, it is not so osmotically active per unit of weight as are the inorganic salts. Using intravenous glucose and judging blood dilution by hemoglobin and specific gravity, Ewing (38) obtained a hydremic plethora, a phenomenon originally observed by Starling in 1899 (39) and verified recently by Barbour and Howard (40). Theoretically, urea should stimulate a similar response.

Without fluid. One gram of urea per kilogram of body weight injected intravenously in the form of a 40 per cent solution does not markedly diminish blood concentration. The hemoglobin curve runs only slightly below the normal, yet it is evident that a mild dilution is present. It is very peculiar that urea may accumulate in the blood without causing a retention of water, a hydremia, which is an occurrence that cannot be duplicated by salt retention. This would

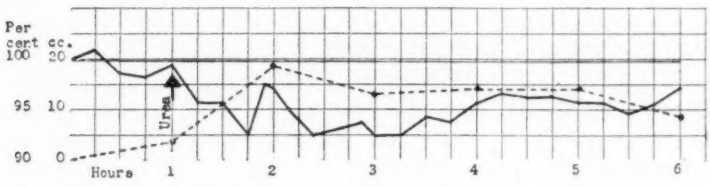

Chart 5. Dog. 1. Weight, 6.6 kgm.; 16½ cc. of 40 per cent solution of urea intravenously (1 gm. per kgm.). ———— Hemoglobin. ---------- Urine output (by hours).

tend to prove that the insignificant blood dilution could not be explained by an exodus of the urea from the blood vessel into the tissue spaces, even though this undoubtedly happens in some degree. Hewlett (41) cogently explains the cause of this difference between sodium chloride and urea as dependent upon a difference in their diffusibility through animal membranes; e.g., urea diffuses easily and its concentration in plasma and red blood corpuscles is approximately equivalent, whereas sodium chloride, sodium acetate, etc., are not so readily diffusible, consequently the salt composition of plasma and of erythrocytes differs. The paradox of an increased urea nitrogen of the blood with no or little blood dilution is probably then accounted for by the diffusion of a goodly portion of the urea into the erythrocytes.

The diuresis is remarkably well sustained, being apparent five hours after exhibition of the drug. At the end of the first hour, the urine output is augmented over 350 per cent above the normal. As with sodium acetate, the blood concentration becomes normal before diure-

sis ceases. Urea, being an organic crystalloid, is quite soluble in colloids and hence passes readily through diffusion membranes (42), or membrane-like structures such as the glomeruli. Siebeck (43) maintains that diffusion within the body is regulated by the dispersion of protoplasmic surfaces, which can be changed by surface-active substances. Whether urea is such a substance or not we cannot say. Ostensibly urea causes diuresis by simple osmosis, yet this is accompanied by a decided increase in oxygen intake (44), which points toward a specific stimulation. Urea can exert its diuretic effect on the perfused, isolated kidney without any corresponding increase in the blood flow (45).

The diuretic effect of urea is so prolonged that the tissues can easily compensate by a release of their fluids to the plasma. The aqueous depletion is not severe enough to cause a subsequent blood concentration.

With fluid. The natural supposition that fluid intake enhances the diuresis of urea injection is confirmed by experiments, of which chart 6 is a typical example. Of the glomerular filtrate, urea is one of the least reabsorbable constituents, although Mayrs (46) insists that 50 per cent of the urea is restored to the blood by the tubules of the rabbit. Indeed urea diuresis may be largely renal due to the inability of the tubules to absorb it (Lamy and Mayer). Free water is essential for urea diuresis, but it is questionable whether the urine output is simply the result of fluid intake alone or a summation of urea and water diuresis. It is well known that the addition of other diuretics can hasten the diuretic action of moderate quantities of water, but this effect is not discernible beyond a certain maximum of fluid intake. In comparing chart 6 and chart 2 it is apparent that the rapidity of urine outflow is increased, when both urea and water are administered, over the rate of output when water alone is given. It should be noted here, however, that the water was given per os and the urea intravenously. These facts may assist in understanding the rapidity of diuresis, as exemplified in chart 6.

Addis, Shevky and Bevier, in their researches, conclude that urea and water effects are independent (47). The water may carry the urea along with it through the glomeruli, the rate of flow being so great that there is not sufficient time to reabsorb the urea, consequently its output is increased. Austin, Stillman and Van Slyke (48) have pointed out that the excretion rate of urea in normal men is proportional to the blood urea and to the square root of the volume of urine, provided the latter does not exceed 5 liters per day. The relations are expressed in the formula; "$D = KB\sqrt{U}$, where D represents the rate of urea excretion, calculated as grams per 24 hours; B the blood urea

concentration (gram per liter); U the rate of urine excretion calculated as liters per 24 hours." This places the diuretic action of urea on a quantitative basis. This factor is taken advantage of in therapeutics as in conditions showing nitrogen retention some increase in the nitrogen output may be obtained by increasing the fluid intake (49).

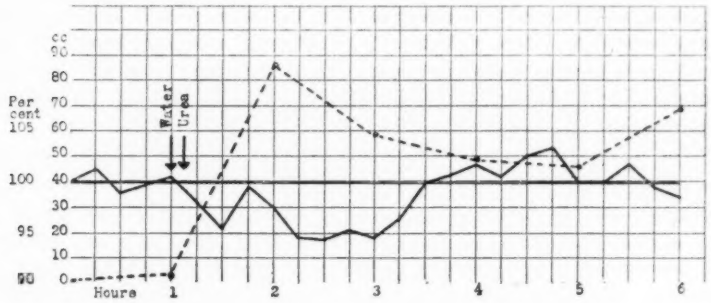

Chart 6. Dog 3. Weight, 5.5 kgm.; 276 cc. of distilled water given by stomach tube (50 cc. per kgm. of body weight); 14 cc. of 40 per cent urea solution intravenously (1 gm. per kgm.). ——— Hemoglobin. ---------- Urine output (hourly).

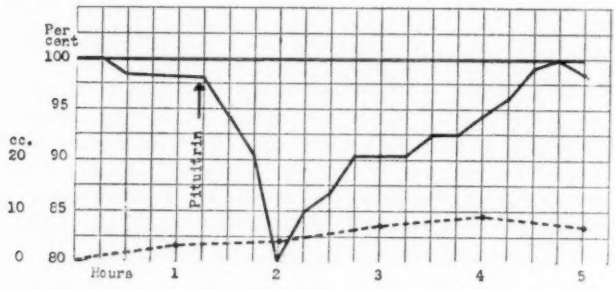

Chart 7. Dog 1. Weight, 7.2 kgm.; 2 cc. of pituitrin intravenously. ——— Hemoglobin. ---------- Urine output (hourly).

The blood concentration changes are negligible, the dilution after urea injection being no greater than in those instances when urea alone was given.

THE INFLUENCE OF INTRAVENOUS INJECTIONS OF PITUITRIN UPON HEMOGLOBIN PER CENT AND URINE OUTPUT IN DOGS. Under different influences of water injection and urine output pituitrin may act as a diuretic or antidiuretic. Its application in therapy is well established, but the mechanism of its action is still a source of dispute.

The most striking phenomenon in our experiments was the marked diminution in blood concentration, the hemoglobin per cent dropping to 80 per cent of the normal figure. The blood volume was greatest one hour after exhibition of the drug, the restoration to normal occurring quickly. Konschegg and Shuster (50) first observed this in humans. Although the presence of an excess of pituitrin in the blood is etiologically responsible for a hydremia, yet the absence of the normal internal secretion is not provocative of a blood concentration, if we are to believe Houssay and Mazzocco (51) who demonstrated that the composition of both the blood and urine of operated dogs (hypophysis extirpation) remains the same as in normal dogs.

The blood dilution is instrumental in explaining and refuting certain recently reported investigations. Stoland and Korb (52) believe that pituitrin injections into dogs produced such a marked secretion of urine that the nitrogen content of the blood falls far below normal. We are inclined to interpret this low nitrogen content of the blood as relative, rather than absolute, due to the hemic dilution by pituitrin. The normal urine output of dogs is 2 to 8 cc. per hour, a secretion the volume of which is augmented by pituitrin to only 8 or 20 cc. hourly, surely not a water loss sufficient to cause noticeable blood changes. Scott (53), as a result of his researches, connects a polycythemia with a rise in blood pressure, the latter being causal. This view is incompatible with our discovery that a pressor drug (pituitrin) produces a relative diminution in hemoglobin.

Without fluid intake, the diuresis was consistently present although only of a magnitude of 200 to 300 per cent of the normal, a commonly observed finding (54) in cats (55) and dogs (56). How does pituitrin increase urine output? Early in this investigation the hydremic plethora was considered a factor, but two points arose which questioned this hypothesis; *1*, the blood dilution is much greater than with sodium acetate injection, yet the diuresis is relatively minute; *2*, the plethora existed simultaneously with pituitrin, anti-diuresis. The explanation of these phenomena will be discussed later.

Current opinion accepts pituitrin diuresis as a direct response to vascular changes (57). Richards and Plant's animals (58) under the influence of minute doses of pituitrin had a decreased blood flow through the kidney, while the kidney volume and urine elimination were increased. This combination of events supports the belief that there may be a slight constriction of the efferent vessels, with a resultant increase in glomerular pressure. Our oncometric tracings verify an increase in kidney volume. (See fig. 1.) Urine secretion is dependent not only on glomerular pressure, but also upon the rate of blood flow through the glomerular capillaries. With a dilatation of the kidney vessels

(59), (60) there is an increased blood flow through the kidney and a consequent increase in urine secretion in intact animals and also in perfusion experiments on the isolated kidney (61). Gabriels (62), working on the isolated kidney of the dog, found an increased urine flow without vasodilatation, subsequent to pituitrin injection, hence it has been thought possible that the local widening of the vessels in the kidney was the result and not the cause of the increased functional activity of this organ, and the diuretic action is of the same type as

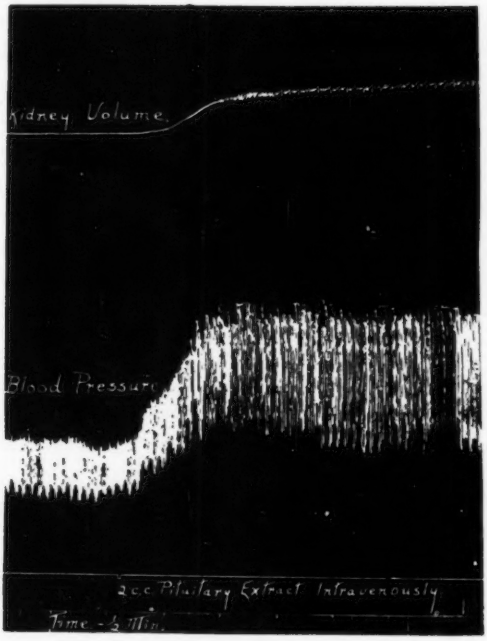

Fig. 1

of caffeine. Not only may diuresis occur without an increased blood pressure, but experimental augmentation of systemic blood pressure does not increase urine flow (63), (64).

Insomuch as there is no constant relation between pituitrin diuresis and systolic pressure, according to Haskins and Means (65), the diuresis is due to direct stimulation of the renal cells. If this is true, pituitary extract must contain another constituent, a diuretic principle, in addition to a pressor principle (66). Knowlton and Silverman (67) deny "that there is any evidence that pituitrin stimulates the kidney cells,

using the oxygen consumption as the criterion, since the oxygen consumption by the kidney is not increased during the diuresis induced by pituitrin."

Not all authors are agreed that pituitrin causes an increased urine secretion; some declare that small doses cause temporary diuresis, followed in a few minutes by diminished urine flow, while large doses diminish the flow from the start (Sollmann). In intact and isolated kidneys (68) of rabbits and cats (69) pituitrin has been said to produce an oliguria (70), which may have a duration of 8 to 10 hours (71). In none of our experiments was this action noted.

With fluid intake. Chart 8 is illustrative of how efficiently pituitrin controls water diuresis. Diabetes insipidus and diuresis from excess water ingestion are probably not etiologically similar, but their response

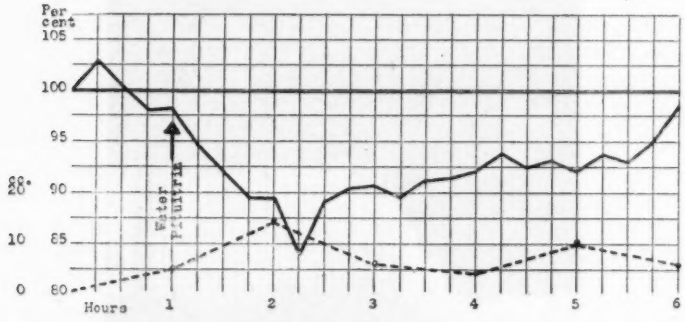

Chart 8. Dog 6. Weight, 10.4 kgm.; 520 cc. of distilled water by stomach tube; 2 cc. of pituitrin intravenously. ———— Hemoglobin. ---------- Urine volume (hourly).

to pituitrin is identical (72). Insomuch as diabetes insipidus is the result of a certain type of hypopituitarism, the hypophysis has been assumed to exercise a regulating influence on the functions of the kidney. Christie and Stewart (73) consider the possibility that in diabetes insipidus there is a lessened tolerance of the renal excretory mechanism to an excess of water in the blood, or a diminished capability of the tissues in storing an excess of water.

How does pituitrin control the polyuria of diabetes insipidus and water diuresis? Rees, using modified Moreau intestinal loops in dogs and cats, believes that subcutaneous injection of pituitrin brings about a delay in the absorption of water from the small intestine, but even he admits that the splanchnic vasoconstriction (very slight) and lessened intestinal absorption are not sufficient to account entirely for the delay in the excretion of water from the kidneys (74). Moreover,

necropsy does not reveal a fluid intestinal content. Dixon (82) denies that pituitrin inhibits water absorption from intestinal loops. That the inhibitory effect on urine secretion is circulatory in origin is contended by Dale (75), his evidence being a vasoconstriction of the renal vessels in perfused kidney (stimulation of sympathetic nervous system (76)). Our oncometric tracings are directly contradictory to this latter finding.

One of the most plausible explanations of pituitrin effects, both with and without water administration, is contained in the following quotation from Oehme (77); "Pituitrin inhibits the sensitiveness of the kidney to the hydremic stimulus. Apparently the effect of pituitrin is to make the kidney cells less sensitive to the diffusion pressure of water in the blood." This observation coincides exactly with our data. Corroboratory evidence is furnished by the fact that the subcutaneous injection of pituitrin is followed by a decrease in the urea excreting activity of the rabbit's kidney, although the blood urea concentration is higher than normal (78).

We believe that the mechanism of pituitrin diuresis and antidiuresis are identically the same. It is only the grossly discernible, misinterpreted phenomena which lead us to classify falsely renal pituitrin action as stimulative and inhibitory. A closer inquiry into the intermediary blood volume changes will probably be helpful in clearing up the mystery. Pituitrin causes a marked hydremia. The so-called pituitrin diuresis is not a diuresis, strictly speaking, if we should compare the pituitrin affected kidney to a normal kidney under similar blood concentration conditions (hydremia). A normal acting kidney, with the stimulus of such a marked hydremia, would secrete four or five times the volume of urine that is put out by the kidney under pituitrin control. By simple comparisons of renal excretion alone, pituitrin does increase the volume output of urine, but considering diuresis from the standpoint of renal activity by itself, with blood concentration as a comparative criterion, the pituitrin affected kidney is decidedly less active. Accordingly we agree to some extent with Oehme that, "pituitrin inhibits the sensitiveness of the kidney to the hydremic stimulus." This hydremia may be contributory to the increased kidney volume caused by pituitrin injection.

The hydremic plethora may be compared to a flood, the pituitrin effect on the kidney as a heightening of the dam (increased renal threshold for water) and the so-called diuresis as an opening of the flood gates so that the stream below the dam (urine flow) is greater than the normal overflow, but is not nearly so marked as it would be if the dam had not been heightened.

The antidiuretic effect of one cubic centimeter of pituitary extract in dogs can be overcome by repeated administration of water (79). During water intoxication in dogs, induced by water and pituitrin, Weir, Larson and Rowntree observed no increase in the total blood or plasma volume by the vital red method, nor any constant increase in relative plasma volume by the hematocrit. Yet in diabetes insipidus, subsequent to pituitary administration, they noted an increase in relative plasma volume and a decrease in the molecular concentration (NaCl) of the blood, which findings they interpret as indicating a probable dilution of the blood. This blood dilution has also been noted by Blumgart (80), Konschegg and Shuster (50) and by Priestley.

How to account for the hydremia is a difficult proposition. It is not directly due to the kidney, since the kidney is already overspilling more than the normal urine output, but we can say that the hydremia would probably not occur to such a marked degree, or would be very

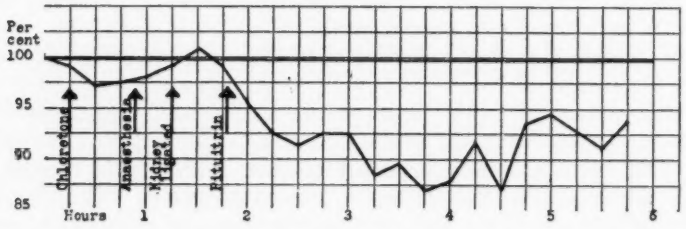

Chart 9. Dog 10. Weight, 10.5 kgm.; 12 cc. of 20 per cent chloretone in alcohol by stomach tube. One hour later—both kidneys ligated. Later—pituitrin (2 cc.) injected intravenously. ———— Hemoglobin.

transient, if the kidney cells were functioning as normal. Rather should we look to capillary permeability changes. In two later experiments the kidneys were ligated and the effects of pituitrin studied. Anesthesia was induced by chloretone. A mass ligature at the hilus of each kidney included renal artery, vein and ureter. Blood volume apparently is temporarily unaffected by anesthesia, or by the exclusion of the kidneys and the consequent loss of their excretory functions. The intravenous injection of 2 cc. of pituitrin, one hour subsequent to renal ligation, was followed in both instances by a relative diminution in hemoglobin per cent (chart 9). This is identical with the blood concentration findings with the kidneys intact.

The simple exclusion of the kidneys by pituitrin does not account for the hydremia, since renal ligation alone does not dilute the blood. It will be noticed that the blood concentration in chart 8 returns very slowly to within normal limits. Could water ingestion delay this

tendency to normal restoration? Probably not, insomuch as *a*, water ingestion alone does not cause blood dilution, and *b*, a hydremic plethora would tend to delay intestinal absorption. Moreover, the same persistence of hydremia occurs with pituitrin administration alone.

Lamson (83) claims that the liver plays an important rôle in the fluid regulation of the blood. By injecting 25 cc. of 0.8 per cent NaCl per kilogram of body weight, intravenously, within ten minutes, he was able to discern that a simultaneous introduction of pituitrin caused a great increase in the time taken for the blood concentration curve to return to normal, even greater than after the removal of the liver. According to Lamson, pituitrin holds the fluid injected within the circulation for over three hours, a duration equivalent to that of our hydremia. He suggests a diminished permeability of the capillaries

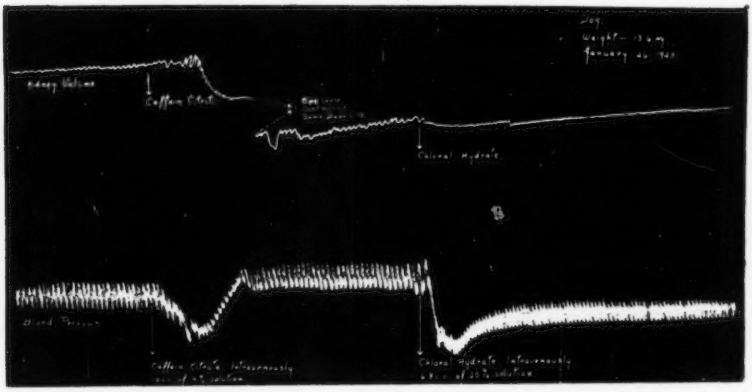

Fig. 2

as the causal factor. This does not explain the initial production of hydremia in our animals.

The antidiuresis is quite persistent. Priestley (81) states that the diuresis produced by water drinking is delayed for four to six hours by pituitrin injection, but after this specific effect wears off, the delayed water diuresis then sets in.

THE INFLUENCE OF INTRAVENOUS INJECTIONS OF THE VARIOUS PURINE DERIVATIVES UPON HEMOGLOBIN CONTENT AND URINE OUTPUT IN DOGS. The three xanthine compounds, caffeine, theobromine and theophylline are without doubt the peers of drug diuretics for man. The rabbit likewise is quite responsive, with diuresis, to the influence of these compounds. The dog, to the contrary, is practically unaffected diuretically by the ingestion of these drugs, especially caffeine. Cushny

quotes Schroeder, Loewi, Fletcher and Henderson as observers of a blood concentration subsequent to and in consequence of an effective diuresis in susceptible animals. By conjecture we are inclined to think that this blood volume diminution would not be marked, and that it would speedily be returned to within normal limits by tissue fluid permeation. That blood concentration follows from caffeine action in susceptible animals (10 per cent in rabbits—Schroeder) answers one of our primary questions: "Does an effective diuresis produce a concentration of the blood?" The fact that dogs are relatively unresponsive to the diuretic action of the caffeine derivatives does not preclude their utilization for experimentation. Such a study may enlighten us as to the occasional, anomalous antidiuretic action of caffeine.

The consensus of pharmacological opinion is that the caffeine derivatives owe their diuretic action to an increased permeability or filtrability of the glomerular capsule to blood serum. This was Schroeder's original contention. It may occur only with the passage of caffeine through the glomerular capsule, if we were to believe Rost (84). He states that the augmentation of urine output only occurs when considerable caffeine passes into the urine. Circulatory changes, especially of the renal vessels, attributed to caffeine action, although not causal, yet are valuable adjuncts to diuresis. Caffeine, however, by its vasoconstriction action with large doses, may antagonize or overcome its diuretic tendencies. This is particularly noticeable in the dog.

Widmer has made the statement that the efficiency of caffeine as a diuretic is proportional to the amount of body fluid (85). This is undoubtedly true, if we recall clinically that caffeine acts best when the tissues are water-logged. Other investigators have demonstrated that dry-fed, water-deprived rabbits do not respond well to caffeine derivatives. Apparently, then, caffeine only facilitates or hastens the excretion of water. Consequently in the following experiments caffeine administration was attended with and without fluid intake.

As can be seen in charts 10, 11 and 12, the laboratory dogs did not show an increased urine output subsequent to the intravenous injection of citrated caffeine, diuretine or theocine. This phenomenon has its clinical exemplification in the failure of caffeine diuresis in experimental nephritis (86), and acute and chronic nitrogen retention (87). The reasons for the non-diuresis in these instances are probably not identical.

Insomuch as there was no diuresis following the injections of these three drugs, there was also no increase in the percentage of hemoglobin content. Throughout these and the remainder of the experiments, caffeine, theobromine and theophyllin behave approximately the same.

Of the innumerable theories submitted as explanatory of caffeine diuresis, none have been found that cite a hydremia as the modus operandi. As the result of a series of perfusion and ultrafiltration experiments in frogs, Ellinger, Heymann and Klein (88) ascribe the diuretic action of caffeine to its effect upon the blood serum proteins in rendering them less hydrophilic and thus increasing the free water available for kidney secretion. The hemoglobin level in our experi-

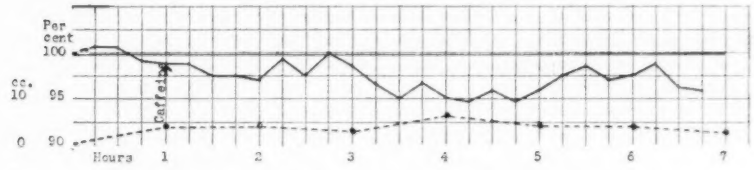

Chart 10. Dog 1. Weight, 7.2 kgm.; 12 cc. of 2 per cent citrated caffeine solution intravenously. ——— Hemoglobin. ---------- Urine volume (hourly).

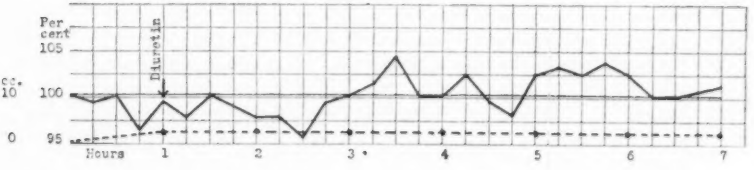

Chart 11. Dog 2. Weight, 5 kgm.; 4 cc. of 10 per cent diuretin subcutaneously ——— Hemoglobin. ---------- Urine output (hourly).

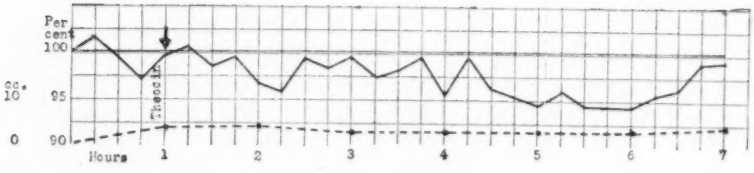

Chart 12. Dog 1. Weight, 7.2 kgm.; 3 cc. of 4 per cent theocin intravenously. ——— Hemoglobin. ---------- Urine volume (hourly).

ments remained within the limits of normal variation, both with and without fluid intake.

How does caffeine administration influence water diuresis? Sollmann and Hofmann (89) maintain that once an effective diuresis has been obtained by an excessive water ingestion, that the urine output is not augmented by the addition of caffeine. The remarkable results in our experiments were due to the ability of intravenous caffeine to inhibit water diuresis (see charts 13, 14 and 15). In these instances,

the rather large dosages of the purine derivatives used were injected intravenously at a time immediately after the ingestion of water per os (50 cc. per kilogram). We know that occasionally the intravenous

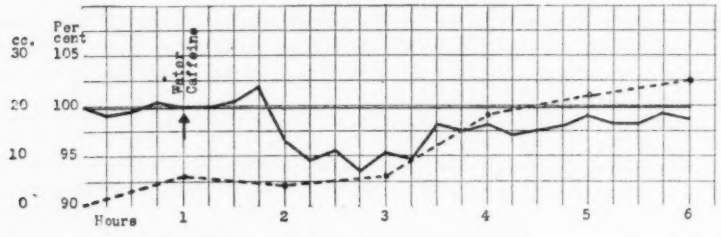

Chart 13. Dog 3. Weight, 6 kgm.; 300 cc. of distilled water given by stomach tube; 10 cc. of 2 per cent solution of citrated caffeine intravenously. ——— Hemoglobin. ---------- Urine output (hourly).

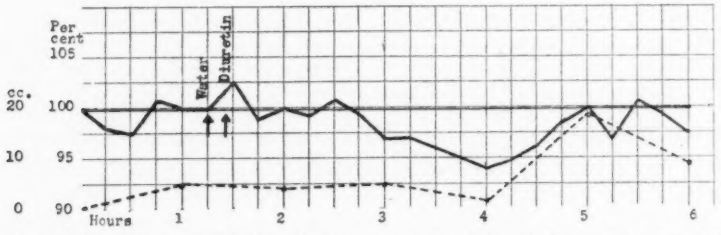

Chart 14. Dog 6. Weight, 10.8 kgm.; 540 cc. of distilled water by stomach tube; 5½ cc. of 10 per cent solution of diuretin intravenously. ——— Hemoglobin. ---------- Urine output (hourly).

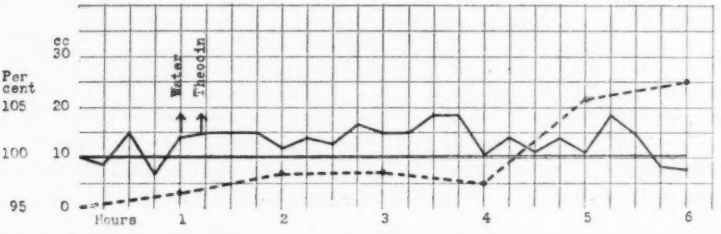

Chart 15. Dog 5. Weight, 11.8 kgm.; 590 cc. of distilled water by stomach tube; 5.9 cc. of 4 per cent solution of theocin intravenously. ——— Hemoglobin. ---------- Urine volume (hourly).

injection of caffeine is followed by a temporary diminution in urine output, but this is the first time that we were aware that this inhibition is sufficient to prevent the natural diuresis that follows the ingestion of such large quantities of water.

The question now assails us: How does caffeine inhibit water diuresis? There are many possibilities. Does caffeine prevent the absorption of water from the intestine? Does it impair capillary permeability? Does it prevent the secretion of urine by renal circulatory changes or by rendering the glomerular capsule relatively impermeable?

Fleisher and Loeb (90) have shown that caffeine lessens osmotic absorption in normal animals. With this experiment in mind, we tested the absorbability of the intestine under caffeine administration. Dogs were the experimental animals. Ether anesthesia was used, because it does not modify osmotic absorption. The small intestines were opened, cephalad and caudad, and the lumen flushed with warm normal saline solution. Then modified Moreau loops were made, two in number, by ligating the intestines. The following protocol is self-explanatory.

Dog. Weight, 10 kgm. Distilled water used

	Loop A		*Loop B*
3:15 p.m.	200 cc. Introduced	3:22 p.m.	210 cc. Introduced
4:00 p.m.	145 cc. Withdrawn	4:07 p.m.	175 cc. Withdrawn
	55 cc. Absorbed		35 cc. Absorbed
4:20 p.m.	Gave 6 cc. of 4 per cent theocin intravenously		
4:27 p.m.	210 cc. Introduced	4:33 p.m.	215 cc. Introduced
5:12 p.m.	150 cc. Withdrawn	5:18 p.m.	140 cc. Withdrawn
	60 cc. Absorbed		75 cc. Absorbed

The above experiment demonstrates that caffeine does not diminish the absorption of water from the intestines. Although this absorbed fluid is not secreted by the kidney, yet it is not held within the blood vessels but finds its way into the tissue spaces. The hemoglobin level is unchanged. Gaisböck states that caffeine diuresis does not affect the restoration of blood after hemorrhage, so he infers that capillary permeability is unaltered by caffeine (91).

Any drug that stimulates in small doses has a tendency to depress by irritation in large doses. We do not believe that caffeine impairs the glomerular capsule permeability, because an irritation of this degree would not be likely to be overcome after three hours, as in these cases. The antidiuresis gradually gives way after three hours and the delayed water diuresis then sets in. Rather must we look to circulatory changes as the responsible agencies.

In considering the circulatory effect of caffeine as the possible antidiuretic factor, two possibilities are presented. By vasoconstriction of the renal vessels, the quantity of blood perfusing the glomerular capillaries may be so diminished that the urine output falls. The cardiac depressant effect of intravenous caffeine in large dosages may so lower

the blood pressure that the urine output is diminished. Now when excised organs are perfused with caffeine (92) the blood vessels tend to dilate, but this does not cause diuresis in the intact kidney. It is well recognized that dilatation of the renal vessels may occur during caffeine diuresis (93), but this is more likely due to the augmented kidney activity than to the direct effect of the caffeine. Richards and Plant (94) found that an increase in kidney volume was not a necessary accompaniment of caffeine diuresis. Sollmann finds in excised kidneys that vasoconstrictor drugs diminish and vasodilator drugs augment the urine flow. That intravenous caffeine in small doses can cause a vasoconstriction has been proven by Sollmann and Pilcher, who noted a diminution in kidney volume while the blood pressure remained level. Salant (95) found that 15 to 25 mgm. of caffeine per kilo injected intravenously in animals produced a transitory fall of 7 to 37 per cent in blood pressure. This fall has been attributed to cardiac depression, and is accompanied by a drop in the oncometric curve; as the blood pressure recovers the kidney volume recovers in a parallel curve, according to Gottlieb and Magnus (96). Analysis of our kidney volume experiments and tracings show that they at least do not differ from this behavior.

Yet there are certain facts which leave one in doubt as to whether this cardio-vascular depression suffices as the sole cause of the inhibition of water diuresis. One of these is the ready response of the caffeine-affected kidney to the antagonistic influence of chloral. Another reason is the comparative length of time of the diuresis inhibition and the blood pressure diminution. Although a fall in pressure does occur, it is fleeting and returns to normal within a few minutes, whereas diuresis is delayed for three hours.

Chloral has been prescribed with caffeine and with digitalis as a "corrective" to overcome their renal vasoconstrictor effects (26). Dixon (97) states that caffeine excites the vasomotor center of the medulla and so constricts the blood vessels. Chloral presumably antagonizes the stimulating action of caffeine on the vasomotor center. In animals sensitive to the diuretic action of caffeine, chloral hydrate acts synergically by a summation effect of the vasodilator actions of these two drugs (98). In the experiments reported here, the dogs exhibited no diuresis following the intravenous injection of caffeine citrate with chloral hydrate. Evidently the natural idiosyncrasy of the dog to the renal action of caffeine is not an increased susceptibility of the renal vessels to caffeine's vasoconstricting property (see chart 16). However, the restraint of water diuresis peculiar to intravenous caffeine injection in the dog is readily overcome by chloral so that a marked augmentation of urine output follows the ingestion of water

per os, a volume output as great or even greater than the controls of water diuresis alone (chart 17). Now chloral hydrate in itself does not directly contribute to a diuresis, because the urine secretion has been shown to be diminished during chloral narcosis (99). The phenolphthalein excretion by the kidney is likewise less when the animal is narcotized by chloral (100). Moreover Sollmann (31) declares that

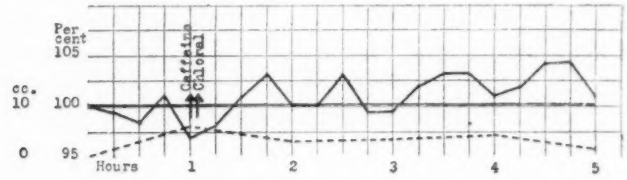

Chart 16. Dog 5. Weight, 10 kgm.; 8 cc. of 4 per cent solution of citrated caffeine intravenously; 5 cc. of 20 per cent solution of chloral hydrate intravenously (0.1 gm. per kgm.). ———— Hemoglobin. ---------- Urine output (hourly).

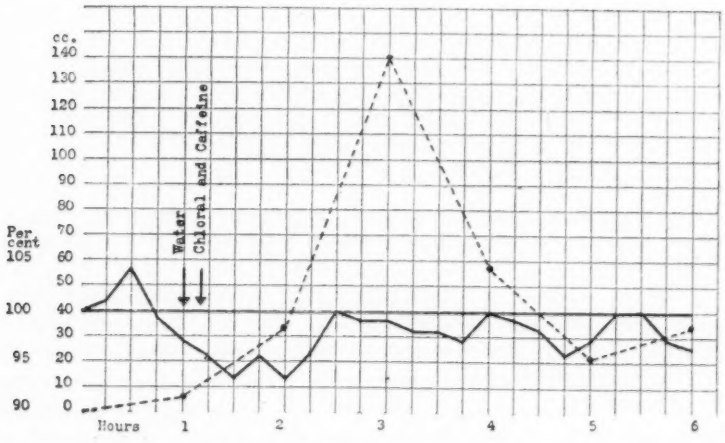

Chart 17. Dog 3. Weight, 6.1 kgm.; 305 cc. of distilled water by stomach tube; 3 cc. of 20 per cent solution of chloral hydrate intravenously; 5 cc. of 4 per cent solution of citrated caffeine intravenously. ———— Hemoglobin. ---------- Urine output (hourly).

chloral may depress the medulla sufficiently to cause a fall of blood pressure. Hence it is fairly well proven that chloral is a depressant to vasoconstriction and to renal secretion; the antidiuretic action of caffeine in the dogs, as manifested by a three-hour delay in the excretion of orally ingested water, cannot be attributed to cardiac depression with its consequent fall of blood pressure, because the addition

of chloral to this condition would simply cause a greater circulatory depression. But, insomuch as the cardio-vascular depressant action of chloral successfully reinstates the ability of the kidneys to excrete this water, we must then indirectly assume that caffeine inhibits the diuretic response of the kidneys to orally ingested water, by causing a vasoconstriction of the renal arterioles.

Schroeder (101), who originally noted the combined effects of caffeine and chloral, found theobromine effective without chloral. In the experiments herein reported, theobromine was as vasoconstricting and antidiuretic as caffeine. It would be interesting to see if the nitrites would be equally successful by their vasodilator property in antagonizing the renal vasoconstriction of caffeine.

One experiment was performed to determine if the caffeine derivatives, intravenously injected, could inhibit the saline diuresis of intravenous 40 per cent sodium acetate solution. Schlosser (102) has

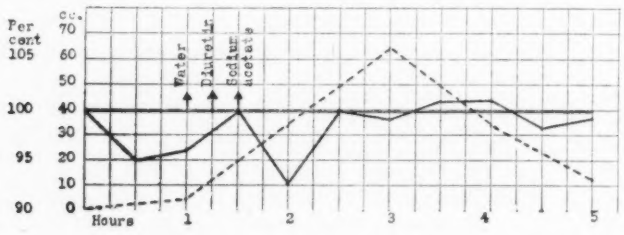

Chart 18. Dog 3. Weight, 6.2 kgm.; 310 cc. of water by stomach tube; 6 cc. of 10 per cent solution of diuretin intravenously; 15½ cc. of 40 per cent solution of sodium acetate intravenously. ——— Hemoglobin. ---------- Urine output (hourly).

shown that saline solutions plus caffeine provoke a greater diuresis than either given alone. In our experiment the saline diuresis occurred notwithstanding the diuretine administration. We have mentioned elsewhere that intravenous hypertonic saline solutions augment the kidney volume by an increased blood-vessel dilatation. Whether this factor or the strength of the hydremic stimulus is the counteracting agency, we are not at present prepared to state.

THE INFLUENCE OF STROPHANTHIN (INTRAVENOUSLY INJECTED) UPON HEMOGLOBIN CONTENT AND URINE OUTPUT IN DOGS. The natural beta glucoside strophanthin was chosen as the type of digitaloid drug for study, so as to insure uniformity in our method of administration, namely, the intravenous route. Digitalis first made its debut in therapeutics as a diuretic. The consensus of modern pharmacological opinion is that the diuretic action of the digitilas series is the secondary or indirect sequel to the primary effect of a circulatory improvement.

Digitalis increases the cardiac output, augments the pulse pressure and thereby is responsible for the absorption of the effusions and edema of cardiac dropsy.

The digitaloid drugs are not diuretic in normal individuals (Schwartz, quoted by Sollmann). Recently Cushny and Lambie (103) have verified this negative result for strophanthin. As long as the circulation is intact, the kidney enjoys no greater facilitation of urine secretion during strophanthin administration. This is the direct antithesis of the opinion that strophanthus has a direct stimulating effect upon the kidney and is superior to digitalis as a diuretic (104).

Physiological normal doses were given intravenously to dogs without any noticeable effects upon the quantity of urine secreted or upon the hemoglobin level. Although such a dose is not diuretic, it does not prevent the excretion of the normal amount of urine. By error, a

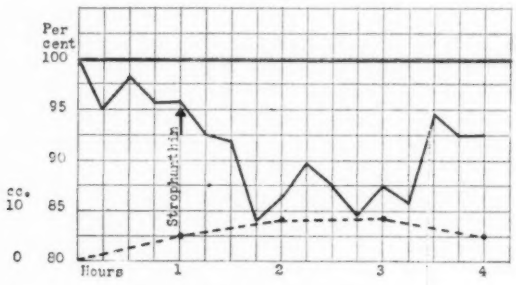

Chart 19. Dog 3. Weight, 6 kgm.; ½ cc. of $\frac{1}{10}$ per cent solution of strophanthin intravenously. ——— Hemoglobin. ---------- Urine volume (hourly).

dog was injected with a toxic dose (ultimately lethal) of strophanthin, and chance observation in this case showed a blood dilution. Consequently another series of experiments was performed with toxic doses of strophanthin, and the majority of these presented similar findings to that cited above.

When strophanthin is indicated as a diuretic, e.g., in cardiac dropsy, it is to be expected that a hydremic plethora occurs, because of the absorption of the dropsical fluid, but this fact does not contribute to the cause of the blood dilution in normal animals. Toxic doses only of strophanthin produce this phenomenon, hence it might be inferred that the ensuing circulatory depression is in some way linked with this action. McLean (105) found a decreased concentration of chlorides in the plasma after the action of digitalis, apparently resulting from a temporary lowering of the chloride threshold. This might have been a dilution effect. Loria (106), on the other hand, maintains that

strophanthin increases the viscidity of the blood even in instances when a diuresis is not present. The presence of a hydremia without a concomitant diuresis can be attributed to the circulatory depression.

Kidney volume during strophanthin toxicosis was not determined, but the study of urine output contributes important information. Several investigators, Cushny, Loewi and Jonescu (107) and others note that there is renal vasoconstriction with toxic doses of digitalis; however, strophanthin is reputed to be less vasoconstricting than digitalis. Naturally with a diminution in the renal blood supply there would be a consequent fall in urine output (97). Although we have no positive evidence that vasoconstriction does not take place, yet we can conclude from our objective findings that urine flow is not

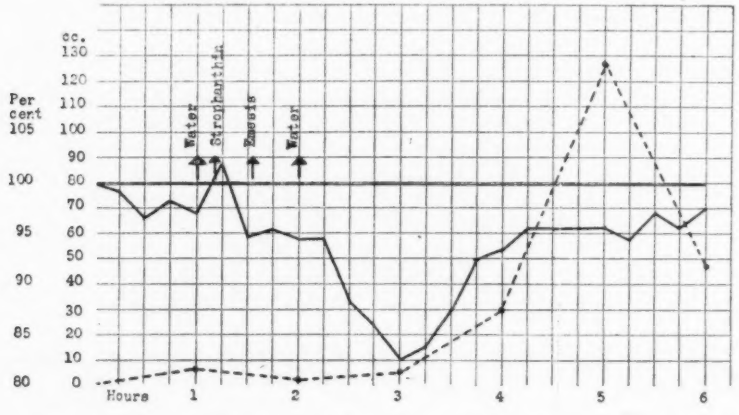

Chart 20. Dog 3. Weight, 5.4 kgm.; 270 cc. of distilled water by stomach tube; ½ cc. of $\frac{1}{10}$ per cent strophanthin intravenously. Severe emesis. Practically all of previously administered water vomited; 260 cc. of distilled water again given by stomach tube. ——— Hemoglobin. ---------- Urine volume (hourly).

diminished by the injection of toxic doses of strophanthin. Not only is the normal urine output the same, but the kidneys are still susceptible to the stimulus of absorbed water, and respond with a water diuresis, subsequent to water administration per os.

ADDENDUM. One of the questions we asked ourselves in the early part of this paper was: "Is diuresis possible when the blood is concentrated?" None of our experiments had a bearing on this interesting problem. Recently, Levy (108) has shown that intravenous injections of calcium chloride are followed by blood concentration. This concentration not only continues and increases during the diuresis which occurs, but even precedes the increased urine output. Hence

we could conclude from this that the concentration is not the result of the diuresis. Additional proof of this is that after diuresis, a dilution of the blood occurs.

SUMMARY AND CONCLUSIONS

1. In dogs, diuresis is not followed by blood concentration.

2. Blood dilution alone does not insure an increased kidney secretion.

3. The oral ingestion of 50 cc. of water per kilogram of body weight does not measurably dilute the blood, although a marked diuresis may be present.

4. The intravenous injection of sodium acetate (1 gram per kgm. of body weight) in hypertonic 40 per cent solution produces a quite marked hydremia, which is concomitant with, but of shorter duration than, the accompanying saline diuresis. Hemoglobin percentage returns to normal within 90 minutes.

5. Water ingestion per os in quantities mentioned above, increases the blood dilution induced by intravenous hypertonic sodium acetate solution. This is attributed to a quicker absorption of fluid from the intestine, due to the increased osmotic attraction of the hypertonic blood. The diuresis is correspondingly magnified.

6. Intravenous urea injections provoke a ready diuretic response. The blood dilution is so slight, that the diuretic activity might be attributed to the inability of the renal tubules to absorb urea.

7. Pituitrin injected intravenously is responsible for a marked diminution in blood concentration. The slight augmentation of urine secretion is not proportional to the extent of the hydremia present. Using the blood concentration as a standard for comparison, the pituitrin affected kidney is less sensitive than the normal kidney to the hydremic stimulus; hence pituitrin is not diuretic but relatively antidiuretic. Renal ligation does not modify the production of hydremia by pituitrin. Pituitrin inhibits water diuresis. The antidiuretic action and hydremia are probably due to some, as yet unexplainable, action on capillary permeability.

8. In the dog, the intravenous injection of the various caffeine derivatives does not increase the volume of urine output. These same drugs produce no change in the percentage of hemoglobin. Intravenously injected and with suitable doses they inhibit water diuresis, probably by renal vasoconstriction. By its vasodilating action, chloral overcomes this inhibition of water diuresis, but does not remedy the canine idiosyncrasy of a negative diuretic response to caffeine injection. The caffeine derivatives do not delay absorption of fluid from the intestine. The inhibition of kidney secretion is not strong enough to overcome the diuretic force of intravenous hypertonic saline injection.

9. Toxic or lethal doses of intravenous strophanthin cause a diminution in blood concentration, but no increase in urine flow. The absence of a diuresis in the presence of a hydremia is probably due to circulatory depression. The kidneys are still susceptible to the secretory stimulus of absorbed water.

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THE NATURE OF THE DELAY IN THE RESPONSE TO THE SECOND OF TWO STIMULI IN NERVE AND IN THE NERVE-MUSCLE PREPARATION

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INTRODUCTION. In 1909 Keith Lucas (1) reported that when two stimuli were applied directly to the sartorius muscle of the frog, the second stimulus occurring at various intervals after the end of the "refractory period," the second electric response followed its stimulus with a greater delay than did the first. His experiments indicated that if the second stimulus was applied at any time between the end of the "refractory phase" and the time at which it was able to evoke a response without delay, the second response occurred at a constant interval after the first response. This type of relation between the time of the second stimulus and the time of the resulting response is shown in figure 1A, in which the method Lucas himself used to express

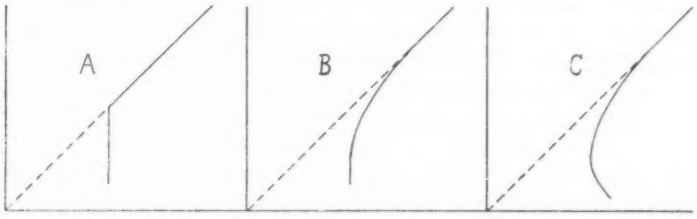


Fig. 1. Schematic curves showing three possible types of correlation between time of second stimulus and time of second response. Abscissae, response-intervals; ordinates, stimulus-intervals. (See text.)

this relation is adopted. Practically, it amounts to the plotting of intervals between stimuli as ordinates and intervals between responses as abscissae, although in his curves the actual abscissa is the interval between the first stimulus and the second response, and therefore differs from the interval between responses by the latency of the first response. As a result of these experiments he proposed the term "irresponsive period" for the period between the first response and the constant time thereafter at which the earliest second response occur-

red—in other words, the time during which the tissue appeared to be unable to respond again.

The following year Lucas (2) reported a more extensive investigation of the same phenomenon in which he not only repeated and amplified his experiments on the sartorius muscle directly excited, but also extended his investigation to cardiac muscle and to the nerve-muscle preparation, using the gastrocnemius muscle of the frog excited through the sciatic nerve. In the same paper he analyzed the results previously reported by Gotch (3) on the electric response of nerve to two stimuli, and compared these results with his own observations in the case of muscle. He endeavored to draw general conclusions as to the nature of the delay in all of these cases.[1] In this latter paper he modified certain earlier statements in regard to the sartorius muscle. In the first paper he had stated that "second responses which began later than the fixed moment of commencement of the earliest second responses, occurred with a delay equal to that of the first response" (i.e., with the normal latency). The more careful measurements recorded in his later paper showed that as the interval between the two stimuli is increased there is not, as he originally supposed, an abrupt transition from a fixed interval between first stimulus and second response to a fixed interval between second stimulus and second response. This fact is indicated by the curved portion of the graph in figure 1B; the interval between the first stimulus and second response does not remain constant up to the point where the second response begins to follow the second stimulus with a constant (normal) latency (a condition indicated by the sharp bend in the graph of fig. 1A); but there is instead an intermediate stage in which the second response occurs later than the end of the so-called irresponsive period, yet still after an abnormal delay. Lucas also found this condition in the second response of the heart (ventricle). He also called attention to the fact that Gotch's observations on nerve showed the same type of relation between the time of second stimulus and the time of second response. Fuji obtained similar results with the electric organ of the electric ray (5).

All the cases thus far mentioned are those of simple tissues; that is, in each case (with the possible exception of the electric organ) the tissue whose responses were recorded was directly excited by the electrical stimulus.

[1] In a preliminary note on our experiments (4) we made the statement that Lucas ascribed the delay to slower conduction than normal. A more careful reading of his paper has shown us that he rejected this explanation as inadequate, and remained non-committal as to the way in which the "modification" caused the delay.

Lucas found that in the more complex case of the nerve-muscle preparation in which the nerve was stimulated and the responses of the muscle recorded, there is an apparently different type of correlation between the time of second stimulus and the time of second response. This is indicated in the graph of figure 1C, and it will be seen here that the response curve not only diverges gradually from the stimulus line as in figure 1B, but that as the interval between the two stimuli is decreased beyond a certain point the time of the second response actually becomes later again. He assumed that this latter relation, indicated by the bending back of the curve, was "associated with the passage of the propagated disturbance through successive tissues having unlike time relations." In this paper Lucas gave reasons for abandoning the use of the term "irresponsive period" as being somewhat misleading, and suggested that no special term should be adopted until more was known about the subject.

By way of explanation, Gotch had suggested that the second response was delayed because it was propagated along the fibers more slowly than the first. Lucas pointed out that this would explain the appearance of the second response after a fixed irresponsive period, such as his earlier experiments had led him to believe existed, for if the second response, occurring during the time of modification, should travel more slowly than the first it would lag behind it till the interval sufficed for complete recovery. As Lucas puts it, "as soon as, in consequence of such slowing, it comes to lag so far behind the earlier disturbance as to find each part of the tissue on which it enters just recovered from the modification, then it will no longer be slowed, but will follow the former disturbance at an equal pace. The result of such a mechanism would be that the second disturbance, provided only that it set out before the modification due to the first had subsided, would come to follow the first at a fixed interval equal to the duration of the modification which the first disturbance had set up" (2, p. 390). Lucas further maintained that this explanation failed to account for the gradual divergence of the curve shown in figure 1B, that is, for the occurrence at certain stimulus intervals of second responses occurring later than the fixed minimum time and yet with an abnormal latency. He also disposed of Gotch's suggestion that the delay might be due in part to a so-called "stimulation fatigue," by showing that the exciting current produced no effect on the delay except in so far as it succeeded in setting up a propagated disturbance; in other words, that the delay was caused wholly by the previous propagated disturbance and not at all by the exciting current *per se*.

Lucas speaks of the cause of this delayed appearance of the second response as a modification of the tissue due to the first response. He

raises the question how this delay is effected, saying, "Is it by a slower development of the second local process of excitation, or by a slower propagation of the second disturbance?" By "local process of excitation" he probably does not mean the change which in a later paper (6) he calls the "local excitatory process" as distinguished from the "propagated disturbance" which it tends to evoke, but rather the whole process of initiating a propagated disturbance. Although he definitely established the fact that the delay was not due to the exciting current, as such, but due to the previous propagated disturbance, he seems to have left unanswered the question how the delay was effected. He implies that the modification which causes the delay is the same sort of thing in all four cases,—nerve, muscle, heart, and the nerve-muscle preparation, although he does call attention to the different type of correlation between stimulus-interval and response-interval introduced in the case of the nerve-muscle preparation (fig. 1C), (cf. (2), fig. 10).

In considering the nature of this delay we should bear in mind that similarity of delay in homogeneous tissue, nerve or muscle, on the one hand, and in the heterogeneous tissues of the nerve-muscle preparation on the other, does not prove unity of cause. It does not necessarily follow from a certain similarity of the curves obtained from the two experiments that there is a single and identical cause involved in the production of the delay in all cases.

We have been led to wonder if it is necessary in order to explain the delay, to assume any modification of the tissue other than the refractory phase. Lucas' experiments clearly show that the delay depends on some effect of the propagated disturbance. With Bramwell he also proved (7) that the refractory phase is a result of the propagated disturbance. At the time of his experiments the extent of the relative refractory phase during which the threshold of excitation of the tissue is abnormally high and returns gradually to normal, was not as well known as it is today. It seems worth while to reëxamine this question with a view to seeing whether the delay observed in all these experiments cannot be explained simply as a result of the refractory phase, when we consider all the known features of the recovery and certain essential facts concerning the mode of excitation by means of the local excitatory process.

At the outset we must consider what we mean by refractory phase. Both Gotch and Lucas used this term to designate the time during which a second stimulus may be applied without causing a response. They speak of the refractory period in these papers as a definite time, and the implication is that they mean the absolute refractory period. There is no mention of varying the strength of stimulus. Apparently they selected at the outset a strength of stim-

ulus above maximal value; that is, adequate to excite all the fibers, and used this throughout the experiment. The latest time of incidence of the second stimulus at which it failed to evoke a response was taken to mark the end of the refractory phase. It is well known that a very strong second stimulus will be effective when applied at a much earlier stage in the recovery of the tissue than that at which a stimulus only slightly above the normal threshold must be applied. In other words, the absolute refractory phase is briefer than the total refractory phase as judged by the response to stimuli only slightly stronger than the normal threshold. This was first established by Boycott (8). In subsequent papers Adrian and Lucas (6), (9) have shown that the "absolute refractory phase" occupies less than half of the total period of lowered excitability.

Certain considerations which have come to light since the publication of these early papers by Gotch and Lucas lead us to question whether there is any important significance to the refractory phase in the sense in which they used the term. In the first place, although we are accustomed to regarding an induction shock as an instantaneous stimulus, in reality no induction shock is absolutely instantaneous; even the briefest induced current has a measurable duration (10). In the case of certain types of induction coil a break shock may have a duration as great as or greater than the refractory phase of a nerve at certain temperatures, and a make shock usually lasts considerably longer than the corresponding break shock. It is a familiar fact that the duration as well as the intensity of an exciting current plays an important part in determining its effectiveness as a stimulus (11). It is well established by the earlier work of Lucas (12) that the local excitatory process, upon which the initiation of a propagated disturbance depends, appreciably outlasts the exciting current, if this be terminated abruptly. It was definitely established by Lucas (13, p. 54) that a stimulus applied during the refractory phase, as well as at other times, may leave behind it a local excitatory process which augments the effect of a subsequent stimulus. Let us assume for purposes of argument that an absolute refractory phase, in the sense in which Lucas used the term, does exist and is significant. Let us assume then that a second stimulus is applied during this time. In view of the above considerations of the duration of the induction shock and the fact that the local excitatory process outlasts the exciting current, it should be theoretically possible to set up a local excitatory process which will outlast the refractory phase and succeed in causing a second response. And this should be possible at any time during the refractory phase if the exciting current is made sufficiently intense and of sufficient duration. In substantiation of this view we have the

fact established by the work of several observers (14), (15), (16) that even a single induction shock, if of sufficient intensity and duration, may evoke in a nerve two successive responses.[2] Evidently the effect produced by a single induction shock may last throughout the refractory phase following the first response and set up a second one. This might be regarded as the extreme case of decreasing the interval between the first and second stimuli. In short, if we allow ourselves sufficiently strong and long-lasting induction shocks, the refractory phase, in the sense in which Lucas and Gotch used the term, may be made to disappear altogether. For this reason it seems best to use the term *refractory phase*, not for the time when a second stimulus may be applied without effect, since this time by modification of experimental conditions may be abolished altogether, but for the time during which the tissue is unable to respond; in other words, we propose to use the term in a sense which corresponds more closely with the meaning originally attached by Lucas to his term *irresponsive period* (1).

We propose to consider to what extent the refractory phase as thus conceived can account for the observed phenomena of delayed second response, and to what extent this may depend on slowed conduction and whether any additional modifications of the tissues must be assumed in order to explain the facts. With these considerations in mind we have carried out a considerable series of experiments with both mammalian and amphibian nerves and nerve-muscle preparations.

METHODS IN GENERAL. In our experiments we have used the isolated sciatic nerve of the frog, the sciatic-gastrocnemius nerve-muscle preparation of the frog, the isolated peroneal nerve of the cat, and in cases in which a great length of nerve was desired, the popliteal-tibial nerve of the cat; that is, the popliteal branch of the sciatic dissected from hip to knee and its extension as the tibial nerve, dissected as far as the ankle. We have also used for mammalian nerve-muscle preparations the peroneal nerve and the tibialis anticus muscle of the cat, and in other cases the popliteal-tibial nerve and the external interosseous muscle of the cat. The advantages of this latter preparation and the technique of preparing it have already been described (16). The cats were decerebrated under deep anesthesia.

For stimulation we have regularly used break shocks. When two stimuli were to be employed we have used for the first stimulus a coil 8 cm. long with a removable iron core (coil 1). In some experiments this coil has been used with the core in place, but in the majority it has been used without the core. For the second stimulus we used a

[2] The occurrence of this effect with extremely strong shocks when it is absent in the case of ordinary maximal stimuli, clearly distinguishes it from the dierotic effect described by Gasser and Erlanger (17).

Berne coil 15 cm. long with a fixed iron core (coil 2). Both coils had been calibrated for break shocks in accordance with Martin's scale (18). In all experiments with two stimuli the secondary coils were connected in parallel. With this arrangement when a current is induced in one coil, the other coil, which is idle, by providing a shunt for the current, decreases the strength of the stimulus to the nerve. Therefore, besides the individual calibrations each inductorium was calibrated when the two secondary coils were connected in parallel, and both connected with the nerve. In some experiments with the frog's nerve-muscle preparation we have used three and even four stimuli. In these cases the third and fourth stimuli were obtained from standard coils with fixed iron core, furnished by the Harvard Apparatus Company. These coils have also been calibrated for break shocks, and as with two coils, additional comparative calibrations were made to show the stimulation efficiency of the shock when three secondaries or four secondaries were all connected in parallel with the nerve. All coils were so connected as to deliver descending break shocks. In choosing the strength of the first stimulus, we made a preliminary test before each experiment to be certain the stimulus was maximal, i.e., that it should excite all fibers.

For determining the interval between stimuli we have used a pendulum patterned closely after the original Lucas design (19). The primary currents were broken by the opening of knockdown keys in the path of the long arm of the pendulum. One key is fixed rigidly to the base on which the pendulum is mounted; another is mounted on a horizontal track with a slow motion screw for adjusting its position relative to the first; a third key is mounted on a large steel disc, and its position relative to the other two can be adjusted by rotating the disc on the same axis as that of the pendulum by means of a slow-motion screw. The velocity of the long arm of the pendulum is more than 3 mm. in 1σ at the point where it knocks open the keys; therefore, by careful shifting of their positions the interval between any two of the three stimuli can be adjusted to within less than 0.05σ. A fourth knockdown key is mounted on a stand, and can also be placed in the path of the moving pendulum. Its time of opening can be adjusted approximately to within 0.1σ. In the experiments on the frog's nerve-muscle preparation a moist chamber was used similar to one described by Lucas and already described (20). In this moist chamber it was possible without disturbing the preparation to lead off action currents from either the nerve or the innervated muscle, and to record simultaneously mechanical contractions of the muscle by means of a light aluminum lever. A water jacket permitted control of the temperature in the chamber. In the case of the isolated mammalian nerve

we used a moist chamber consisting of a long horizontal hard rubber box with openings for the insertion of electrodes at various points. In the case of the mammalian nerve-muscle preparation the tissues were left as far as possible *in situ* in order to maintain the normal body temperature throughout. The method of doing this has already been described (16). For leading-off electrodes we have regularly used the silver chloride type, since these can be more sharply localized on the tissue and more conveniently applied when space is limited than can the porous boot electrodes. For recording action currents we have used a Hindle galvanometer with a 1.5 mm. air-gap. In the course of the research four different gilded quartz strings have been employed, varying in diameter from 1.2μ to 2.7μ.

In nearly all of our experiments the galvanometric records were made on motion-picture film with the camera described in a previous communication (21), operating the film at a speed of about 33 cm. per second. In some of our experiments on the responses of mammalian nerves in which accurate time measurements were required, we have wanted a higher speed of film than could be had with this camera. To this end we have used a high-speed camera which has been mentioned (22) but not described. For the speed required it is not feasible to reel the film from one spool on to another. Instead it must be fixed on the surface of a rapidly revolving drum, and the exposure regulated by a shutter. In this camera a strip of film is clipped around the standard drum of a Sandström kymograph, the shaft of which protrudes through the end of the camera, and on the shaft is secured a square block of brass; a hard rubber cylinder which fits over this block has on its outer surface a spiral groove or thread, in the bottom of parts of which are imbedded two copper wires, each connected to a brass collar or slip-ring at the end of the cylinder (see fig. 2). The camera is provided with an electromagnetic shutter whose moving parts have small inertia, and can be pulled open by a strong electromagnet. The circuit which opens the shutter is closed by contact of a metal brush with one of the copper wires in the spiral groove. The length of this copper wire is such that the shutter is held open for a quarter-revolution of the drum. When the same metal brush makes contact with the other copper wire it closes another circuit whereby an electromagnet releases the Lucas pendulum. The copper wire which completes this latter circuit is the first with which the metal brush makes contact, and it is so placed in the groove that the pendulum is released in time to knock the keys open when the shutter of the camera is open. In performing a series of experiments the drum of the kymograph is kept revolving at its full speed giving it a surface velocity of about 1 meter per second; the pendulum is set, and at the desired moment a trigger is pulled which

lets the metal brush fall into the spiral groove. By means of the mechanism just described a complete observation is thus automatically made and the responses recorded on one quadrant of the film. For the next observation the kymograph is stopped, the hard-rubber cylinder is removed from the brass block, rotated ninety degrees, and replaced so that in the next observation the next quadrant of the film will be exposed. In this way four observations can be recorded on a single length of the film. The drum is wide enough to carry two strips of $3\frac{1}{2}$-inch film. By using a screen with a narrow window in front of the camera it is possible to confine the exposure of the film in a single

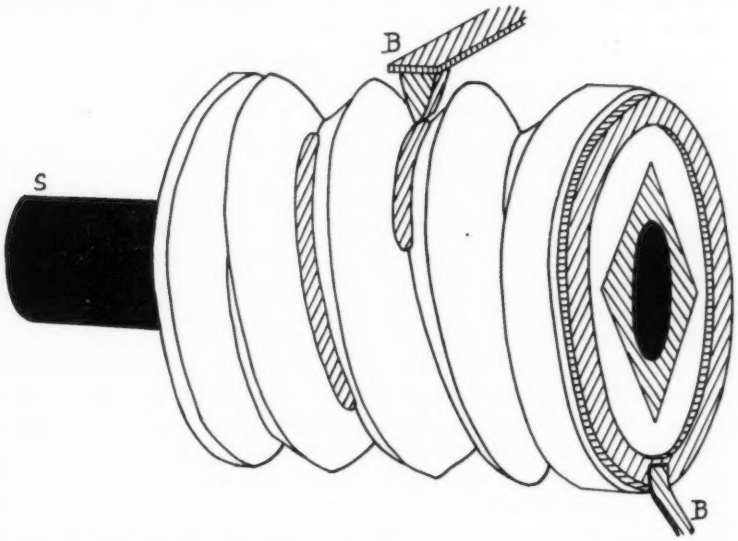


Fig. 2. Mechanism which controls pendulum and shutter of high speed camera. *S*, shaft of kymograph. *B*, *B*, brushes for completing shutter circuit. Metal parts are shown cross-hatched; the remainder is hard rubber.

observation to a width of about 1 inch. By moving the camera after each series of four observations has been made, so that the shadow of the string will fall on an adjoining section, it is possible to take six rows of exposures, making in all twenty-four observations with a single loading of the camera. Because of the permanent installation of the other camera (using motion-picture film) it has been necessary in using the high-speed camera to reflect the image of the string by means of a mirror. The magnification obtained in this way is somewhat greater on account of the extreme distance of the camera from the galvanometer, and the definition is slightly impaired; but the results have

served fairly well for accurate time measurements in some of the experiments (see fig. 12).

A synchronous ordinate, or base line for time measurements, can be made with this camera by bringing the drum to rest with a conveniently placed portion of the film behind the shutter, and then opening this by very brief closure of the circuit. In making time measurements on the film exposed with the other camera, lines caused by a slight flickering of the arc lamp can always be found near enough the observations to serve as synchronous ordinates. Time measurements on the film have sometimes been made with a hand lens and a finely graduated millimeter scale; sometimes they have been made by clamping the film to the stage of a low power microscope and measuring distances by means of a micrometer eye piece. The shadow of a tuning fork provides the standard of time.

Other special procedures will be described separately in connection with the several phases of the problem for which they were designed.

THE DELAY IN THE SECOND RESPONSE OF NERVE. Given the fact that an early second stimulus evokes its response in nerve after an abnormal delay and that, however early this second stimulus is applied its response will never appear earlier in the recovery from the first response than a definite minimum time, it would be simple to explain this fact as a result of the refractory or irresponsive period, during which the nerve is unable to respond again. For Lucas has provided us with the essential condition for this explanation, i.e., the fact that a stimulus applied near the end of the refractory phase can set up a local excitatory process which manifests its effect after the refractory phase has passed away, just as can a stimulus at any other time (13, p. 54). Before deciding whether this simple explanation of the delayed second response is adequate we must consider the fate of the local excitatory process set up by the first stimulus when such stimulus becomes effective and initiates a propagated disturbance.

In a previous paper (15) one of us has suggested, in order to explain the setting up of two separate nerve impulses by a single powerful induction shock, that the local excitatory process set up by the shock was so great as to persist, after initiating the first nerve impulse, throughout the resulting refractory phase and initiate a second one. We have found it necessary to modify this view. Doctor Adrian has called our attention to the fact that the recovery curve of threshold of a tissue emerging from its refractory phase is not at all dependent on the strength of the first stimulus. If the local excitatory process persisted with any appreciable intensity after the initiation of the first propagated disturbance, it should augment the effect of an early second stimulus, producing a summation similar to that observed in

resting tissue. By making the first stimulus very strong it should therefore be possible to render effective a second stimulus at a given time during the recovery process when it would otherwise be inadequate; but Bazett has shown that this is not so (23). Varying the strength of the first stimulus has no effect on the threshold of a second stimulus applied during the recovery process, or relative refractory phase. It occurred to us that the result might have been different if the first stimulus had been made strong enough. It might be that a very large increase in the strength of first stimulus was needed to cause an appreciable local excitatory process to persist long enough to make any difference when the second stimulus was applied. It may have been that in Bazett's experiments only moderate variations in the strength of the first stimulus were tried. We therefore attempted to repeat this experiment, varying the strength of the first stimulus up to approximately 30 times the threshold value. In some of our earlier experiments we found what appeared to be evidence that a very great increase in the strength of first stimulus might render effective an otherwise ineffective second stimulus, occurring during the relative refractory phase.

A typical experiment on this point is as follows: Coils 1 and 2 were arranged with their secondaries connected in parallel with the nerve of a frog nerve-muscle preparation. The iron core of coil 1 was in place. The primary current in coil 1 was 0.03 amp.; in coil 2 it was 0.085 amp. The contractions of the muscle were recorded on a smoked drum. The temperature of the preparation was 25.2°C. With coil 1 alone (stimulus no. 1) the break shock was maximal if the coil distance was 110 mm., giving a stimulus of 12 Z units. With coil 1 at 100 mm. giving a break shock of 14 Z followed at 1.4σ by a break shock from coil 2, there was a summated contraction if the second stimulus exceeded 8.1 Z units (coil distance 196 mm.), but none if the second stimulus was below this value. The presence of a summated contraction is proof of a second response in some of the nerve fibers (cf. 8). A single break shock from coil 1 with the coil distance reduced to 0 mm. (180 Z) produced a simple contraction without summation. This stimulus, followed at an interval of 1.4σ by a second stimulus with a coil distance of 198 mm., caused a clearly summated contraction. This result was obtained three times in alternation with control tests in which double stimulation with the same interval and the same coil distance in coil 2, but with a first stimulus of only 14 Z units, caused no summation of contraction. Apparently the large increase in the strength of the first stimulus rendered an otherwise inadequate second stimulus effective.

More careful examination of our method revealed an important source of experimental error in these observations. This error was due to an actual change in the strength of the second stimulus caused by shifting the position of the coil which delivered the first stimulus. It will be recalled that the secondary coils of the two inductoriums were connected in parallel, and both connected with the nerve. In order to make the first stimulus very powerful an iron core was placed in the inductorium used to produce it. When in the alternate tests just described, the first stimulus was made very strong, the secondary coil was pushed in till it completely covered the primary, whereas with the moderate stimuli (only slightly more than maximal) the secondary coil was withdrawn well beyond an overlapping of the primary. The

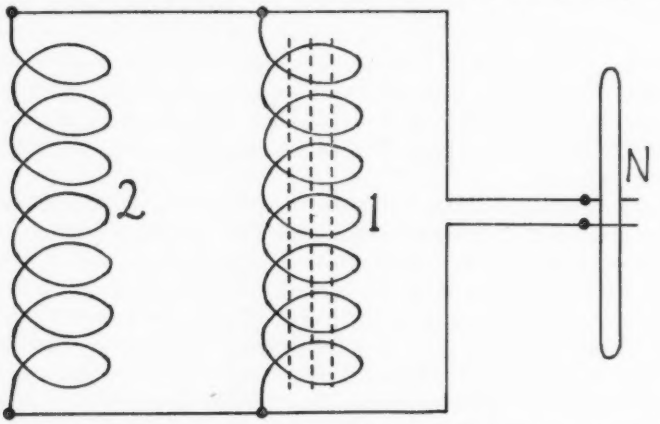


Fig. 3. Diagram showing the secondary coils of two inductoriums connected in parallel with the nerve. *1*, coil used to deliver first stimulus; *2*, coil used for second stimulus; *N*, nerve. Dotted lines indicate movable core in coil 1.

presence of an iron core in the secondary coil increases its inductance and this increases its impedance to the establishment of a current in it by an impressed E.M.F. The secondary coil, being connected in parallel with that of the other inductorium (used to produce the second stimulus), acts as a shunt (see fig. 3); it takes part of the current produced by the second coil and thus reduces the amount of current that passes through the nerve. When the secondary coil has its inductance increased by the iron core its virtual resistance to a current of sudden onset and brief duration, such as an induction shock, is increased; it therefore diverts less current from the nerve, and in this way impairs the efficiency of the second stimulus less than when the iron core is not within the coil. Thus in shifting the inductorium to increase

the strength of the first stimulus we unintentionally increased the strength of the second stimulus at the same time, thereby vitiating the experiment.

As soon as we suspected this source of error we performed a control experiment to determine whether or not the presence of the iron core inside the secondary coil of the first inductorium could be shown to alter the stimuli produced by the second. This was clearly shown to be the case when the stimulus from the second coil was tested by itself, the threshold coil distance in the second inductorium being regularly greater when the secondary of the first inductorium covered the primary with the core in place than when it did not. The strength of the stimulus was changed by this cause in a ratio of approximately 7:5. We also found that with the iron core removed it made no difference to the strength of stimulus delivered by the second inductorium whether the secondary of the first inductorium covered the primary or not; the effect noted above was entirely due to the iron core.

After we had discovered this source of error we removed the iron core from the first inductorium and repeated our experiment to see whether a great increase in the strength of the first stimulus would have any effect at all on the threshold of the nerve during the relative refractory period. We were unable to find any evidence of such an effect, even when the strength of the first stimulus was increased from 4.2 Z to 240 Z. It should be noted incidentally that, judging from the work of Erlanger and Garrey (10), the duration of the break shock was probably decreased by removal of the iron core.

Our results, then, agree with Bazett's in showing that variations in the strength of the first stimulus, provided of course that they are all maximal, have no appreciable influence on the effectiveness of a second stimulus applied during the relative refractory period. The reasons for inserting this confirmation of Bazett's results in detail are,—first, because they have been extended over a very wide range of intensities of the first stimulus, and second, to call attention to the pitfall which an iron core may introduce into this sort of experiment.

The conclusion drawn from these experiments is of interest in connection with the fact mentioned above, that a single powerful shock can set up two successive impulses in a nerve. If this latter fact were due to the local excitatory process, set up by the single strong stimulus, initiating a nerve impulse and then, although the exciting current had ceased to flow, lasting long enough to initiate a second impulse, we should find evidence of this persistence of the local excitatory process in augmentation of the effect of an early second stimulus. This we do not find. Yet we know that when the local excitatory process does not reach the point of setting up an impulse it persists with an appre-

ciable intensity for a measurable time after cessation of the exciting current. This leads us to the inference that when the local excitatory process becomes effective in setting up a propagated disturbance (nerve impulse) it disappears, or at least becomes too small to be detected. On theoretical grounds we might expect this to be the case, for whatever the local excitatory process is, whether an ionic concentration as the Nernst theory holds, or some other change in the state of the colloids which simulates an ionic concentration, it is hardly conceivable that it could initiate the nerve impulse without itself being modified in the act.

Since the local excitatory process can not of itself endure after it has produced its effect, we are forced to the conclusion that the starting of two impulses by a single induction shock must depend on the exciting current itself outlasting the refractory phase. The conclusion seems to be that in setting up a nerve impulse the local excitatory process disappears, but if the exciting current is maintained it will create a new local excitatory process, and if this outlasts the refractory phase a second nerve impulse will result. According to Hill's modification (28) of the Nernst theory, when the local excitatory process reaches the requisite intensity, the ions which have been concentrated by the exciting current react with some hypothetical unstable substance, and this constitutes the initiation of the propagated disturbance. If such a reaction occurs, it must necessarily involve the disappearance of some, if not all, of the reacting ions as such; and this consideration is in harmony with the evidence just adduced to show that the local excitatory process does in fact almost or wholly disappear when it initiates a propagated disturbance.

Let us assume, then, that in order to set up a second nerve impulse, more exciting current must be supplied after the initiation of the first impulse in order to renew the local excitatory process. With this in mind we may return to the question whether we need assume any other modification of the tissue than the refractory period, in the sense in which we propose to use the term, in order to explain the delayed appearance of the second response.

We have already called attention to the fact that even the briefest induction shock has a measurable duration and that the local excitatory process may outlast the exciting current. Therefore, adding these two times together, we have every reason to suppose that a powerful shock applied to a tissue during the time when it is unable to respond, will nevertheless set up a local effect which will persist until the tissue recovers sufficiently to respond again, and will then set up a propagated disturbance. We have in the experiment of Lucas (13, p. 54) definite evidence that a second stimulus applied near the end of the refractory

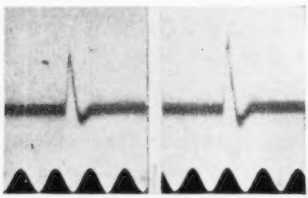
phase, although in itself unable to set up a second propagated disturbance, is able to augment the effect of a subsequent stimulus and cause it to produce a larger second response than it otherwise would. Adrian later demonstrated (24) that during the relative refractory phase the response, although of subnormal magnitude, nevertheless obeys the all-or-nothing law, its size being independent of the strength of stimulus, provided it be adequate; therefore this observation of Lucas is to be interpreted to mean that the second stimulus, although failing in itself to produce any second response, enables the third stimulus to excite a larger number of fibers than it could if the second stimulus was omitted. At all events it demonstrates that during the later part of the refractory phase a local excitatory process can be set up which may reveal itself by the same ability to produce summation then as at other times. At first sight then, it appears that we have all the necessary conditions to explain the delayed appearance of the second response as due to the application of the stimulus when the tissue *cannot* respond, and the persistence of an effect of the stimulus until the tissue *can* respond.

A conceivable objection to this view is that it might lead us to expect the response to a second stimulus, applied at any time before the tissue is ready to respond again, to be delayed to a fixed moment after the first response—a condition which is expressed graphically by the type of curve shown in figure 1A. As Lucas pointed out, the experiments of Gotch showed in the case of nerve, and his own experiments showed in the case of muscle directly stimulated, that this type of correlation of response-interval with stimulus-interval is not found, but instead the more gradual disappearance of delay shown graphically in figure 1B. Before considering a theoretical answer to this objection we shall describe the results of our own experiments on the response of nerve to two stimuli applied at various intervals, in which we have used the nerves of both frog and cat at various temperatures.

In seven experiments on frog nerves and sixteen on the nerves of the cat we have recorded the electric responses to two stimuli applied at such intervals that the second response appeared after an abnormal delay. In four of the experiments on frog nerves and twelve of those on cat nerves we have varied over a fairly wide range the interval between the stimuli. The experiments have been performed at various temperatures, from 11° to 26°C. in the case of the frog nerves, and from 16° to 36°C. in the case of the cat nerves.

We have made no attempt to correlate the absolute time of response with the absolute time of stimulus. We have determined the amount of delay simply by comparing the interval between responses with the interval between stimuli. The interval between stimuli has been

determined by the setting of the Lucas pendulum. This was previously calibrated by connecting its first key with a short-circuit across the string galvanometer, and the second key with a circuit whereby a small current was led to the galvanometer when the short-circuit was open. The interval between responses has been determined by direct measurement on the film upon which they were recorded. In order to facilitate accurate measurement we have used diphasic action currents, placing the two leads fairly close together on the nerve, so that the first phase of the action current would appear in the record as a small sharp spike (see fig. 8). We believe that in this way it is easier to make accurate time measurements of the intervals between responses than when, as the result of a larger space between the electrodes, the galvanometer registers the response with a considerable excursion (e.g., fig. 4). In most of our experiments the leads have been placed 10 or 12 mm. apart.



A B

Fig. 4. Diphasic action currents of peroneal nerve of cat *in situ*. Temperature, 30.3°C. Records enlarged from film. 16,000-ohm gilded quartz string, diameter 1.5μ. Magnification approximately 800. Tension, 39 m. per amp. (See Forbes and Ray (25).) String damped with 0.1 microfarad condenser. (See Einthoven (26).) A, single stimulus, break shock, 10Z. B, two stimuli, interval 0.7σ; 1st stim. 10Z, 2nd stim. 47Z. The second response appears as a thickening of the line in its descending phase. The increased height of the initial excursion in B is probably caused by the electrical artefact from the second stimulus. Time shown by shadow of tuning fork at the bottom; 1 double vibration = 0.01 second.

In measuring the interval between responses on the film we have used two methods. In one we have taken the first excursion of the string as marking the beginning of the first response, and as the beginning of the second response, the first divergence of the curve from one obtained by a single stimulus. The other method was to measure the interval between the peaks of the two responses, assuming that in each case the peak marks the arrival of the impulse at the distal electrode. Lucas has discussed the relative merits of the two methods in connection with corrected capillary electrometer records (2), but in his case the problem was somewhat different in that the peak of the first phase occurred before the disturbance reached the second electrode. The disadvantage of measuring to the first appearance of the second response is that, when this is very small, coming early in the relative refractory phase, the divergence may not be appreciable until a measurable time after it has actually begun. The objection to measuring the interval between the peaks of the responses is that in the case of a small

early second response, whose registration begins with the string already in motion, it may be impossible to tell what point in the curve is the true peak, since we have no accurate method of analyzing the galvanometer records. However, by using a very light string at the optimum tension it is usually possible to obtain a fairly close estimate of the true peak of the curve even in the case of the second response, the lag of the string being so slight that it introduces comparatively little error. In a few experiments at temperatures above 20°C. the action current is so brief and the lag of the string is proportionately so large that the second response appears only as a partial interruption or jog in the second phase of the first response (fig. 4). In such cases we could only measure from the beginnings of the responses. In some cases we have all three made independent measurements, both from the beginnings and from the peaks of the responses, and have averaged our results. In almost every record the measurements have been made by more than one of us. We believe that in almost every experiment the measurement finally agreed on has an error of not over $\pm 0.3\sigma$ and that the average error is much less than this. In some few observations there may be an error as great as 0.4σ, but this is exceptional. It should be recalled that the response and the refractory phase in nerve are both much briefer than in muscle, and that since Lucas' observations were made on muscle, he was able to follow the course of the responses relatively in more detail than we have in nerve. In our case the most accurate measurements, relatively, can be made on frog nerve records, since the temperature of amphibian tissues can be reduced more without introducing abnormalities than is possible with mammalian nerves; and the lower the temperature, the slower are all the processes. A given absolute error in measurement, therefore, will be relatively less at low temperatures than at high.

Experiments on frog nerves. In our four most complete and accurate experiments on frog nerves we have clearly corroborated Gotch's observation that the curve correlating time of second response with time of second stimulus conforms to the type indicated in figure 1B, as in the case of Lucas' later experiments on the sartorius muscle. That is, as the stimulus-interval is increased, we did not find an abrupt transition from a fixed irresponsive period to a normal latency in the time of the second response, but a gradual decrease in latency to normal even after the second response had begun to occur later than the minimum time after the first. With very short intervals between stimuli we also found a condition which Gotch did not find in nerve, and which Lucas did not find in muscle directly stimulated, but only in muscle excited through its nerve, namely, an actual *increase* in the interval between responses as the stimulus-interval was decreased

below a certain point, a condition represented in figure 1C (cf. *2*, fig. 10). Records showing this in two experiments on frog nerves, together with a similar experiment on a cooled mammalian nerve, are reproduced in figure 5. The correlation between stimulus-interval and response-interval is shown in figure 6, in which the results of the same three

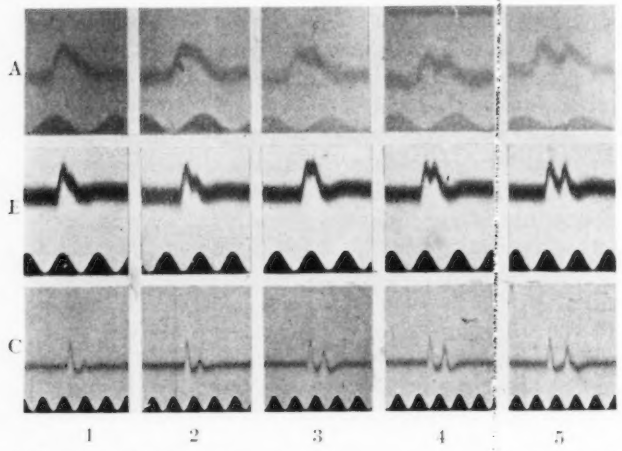


Fig. 5. Records of action currents of nerves in response to two stimuli at various intervals, showing the resulting intervals between responses. Each horizontal line comprises records from a single experiment.

A. February 14, 1923. Frog sciatic nerve. Temp. 22°C. Distance stim. to prox. lead, 30 mm.; distance between leads, 9 mm. Stim. intervals: *1*, 1σ; *2*, 1.3σ; *3*, 2σ; *4*, 3σ; *5*, 4σ; first stim., 10Z in all; second stim., *1*, 46Z; *2*, 38Z; *3*, 27Z; *4*, 32Z; *5*, 27Z. 4,800-ohm string, 2μ diam., tension, 72 m. per amp.; 0.3 microfarad condenser damping. Magnification approximately 700. High speed camera.

B. February 21, 1923. Frog sciatic nerve. Temp. 20.8°C. Stim. to prox. lead, 25 mm. Stim. intervals: *1*, 0.75σ; *2*, 1.3σ; *3*, 2σ; *4*, 3σ; *5*, 5σ. First stim., 28Z in all; second stim., *1*, 61Z; *2*, 61Z; *3*, 42Z; *4*, 35Z; *5*, 25Z. 4,800-ohm string, 2μ diam., tension, 89 m. per amp. Magnification approximately 800. (Enlarged from film.)

C. May 10, 1923. Cat popliteal-tibial nerve. Temp. 16.5°C. Stim. to prox. lead, 18 mm. Stim. intervals: *1*, 3σ; *2*, 4σ; *3*, 5σ; *4*, 6σ; *5*, 8σ. First stim., 22Z in all; second stim., 38Z in all. 16,600-ohm string, 1.25μ diam., tension, 102 mm. per amp. Magnification, 490.

Time in all experiments by tuning-fork shadow; 1 d.v. = 0.01 sec.

experiments are plotted. As in some of Lucas' figures we have indicated the approximate magnitude of the possible error in the results by the size of the rectangles in the graphs. In order to be certain that we were not deceived by a progressive change in the state of the preparation, we covered the range of intervals both forwards and back-

wards in two of these experiments; that is, we tried the longer stimulus-intervals both before and after the shorter intervals.

There seems to be no doubt that the curve really does bend back, expressing an increase in response-interval as the stimulus-interval is decreased to its smallest values. This result does not necessarily imply any contradiction of Gotch's work; it rather supplements it. It is quite possible that if he had tried a stronger second stimulus than he did, at a briefer interval than the briefest at which his second stimuli were effective, he would have obtained a second response occurring later than the minimum irresponsive period, just as we did. We are not prepared to say that in every nerve at every temperature this re-

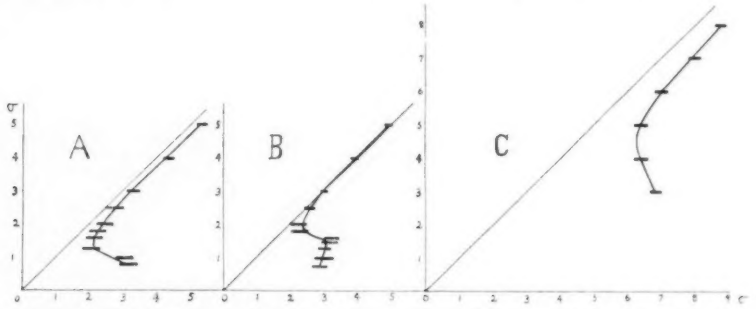


Fig. 6. Graphs of same three experiments furnishing records in figure 5. For experimental conditions, see legend to figure 5. Abscissae, response-intervals; ordinates, stimulus-intervals. A, February 14, 1923; B, February 21, 1923; C, May 10, 1923.

The peculiar shape of curve B probably signifies that some fibers ceased to give a second response when the stimulus-interval was decreased below 1.8σ.

sult can be found. In only three experiments on frog nerve have we searched for it systematically with a strong second stimulus at a very brief interval. In two of these the result appeared; in the third it did not.

Experiments on mammalian nerves. In most of our experiments on cat nerves we have been chiefly concerned with other phases of the problem than mapping out the correlation of response- and stimulus-intervals over a wide range in the early stages of recovery. We have therefore not usually searched systematically for the fine points of the correlation curve, especially since this can be more accurately done with a frog nerve. One experiment in which a mammalian nerve, cooled as much as possible without impairing its responses, was examined in this way, showed the same type of correlation as the frog nerves. This has already been illustrated in figures 5 and 6. At normal mammalian body temperature the intervals involved are so

brief that errors in measurement may simulate or conceal significant changes in the intervals between responses. We have, nevertheless, a few records which seem to show in mammalian nerves at body temperature the same type of correlation. Figure 7 shows the data of three experiments on mammalian nerves at approximately normal body temperatures. In order to give an idea of the degree of accuracy

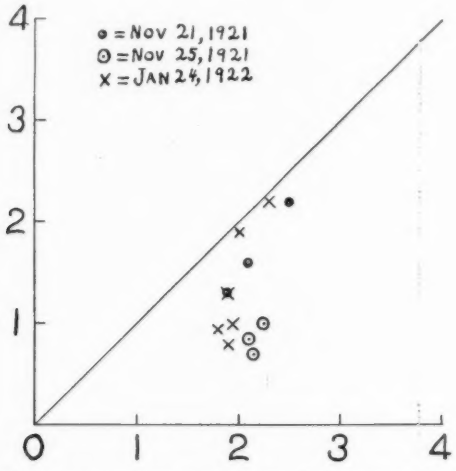


Fig. 7. Data of three experiments on mammalian nerves plotted together by the method of figure 6; abscissae, response-intervals; ordinates, stimulus-intervals.

November 21, cat peroneal nerve in moist chamber. Temp., 32°C. First stim., 52Z; second stim., varied from 38Z to 79Z in different observations.

November 25, cat peroneal nerve *in situ* in body with shielded leads. Temp., 30.4°C. First stim., 10Z; second stim., 23Z to 46Z.

January 24, cat popliteal-tibial nerve *in situ;* distance stim. to leads, about 10 or 12 cm. Temp., 33.4°C. First stim., 10Z; second stim., 14Z to 67Z.

Each point in the graph is the average of measurements by two or three observers on one or more observations,—usually two or three duplicate records under identical conditions. Some of the records appear in figure 8A.

of which the measurements are capable we have reproduced in figure 8 some of the records made in experiments of this group. As Lucas has insisted (2, p. 387), it is not valid to make a single curve from the data of two or more separate experiments of this type, since variations between individual nerves may suffice to vitiate the comparison involved. "The observations on each preparation must stand alone." Yet the properties of homologous nerves in cats at the same temperature are probably nearly alike; and it may be useful to plot the results

of three similar experiments together, using special means for differentiating between them. By so doing, as we have in figure 7, we may perhaps bring out better the trend of the correlation curve in question. It should be noted that the observations plotted in this figure include responses to second stimuli of various strengths. It will presently appear that this probably has some influence on the result, but the change in latency from this cause is not enough to obscure the true correlation, and the figure as it stands distinctly suggests that the type of correlation shown in figure 1C and in figure 6 is general for the

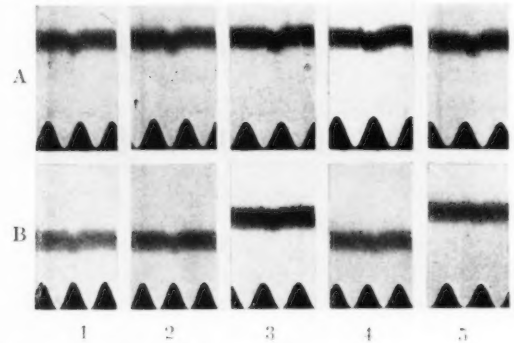


Fig. 8. Galvanometer records from mammalian nerves *in situ*,—cat popliteal-tibial nerve.

A. January 24, 1922. Temp., 33.4°C. *1*, first stimulus alone, 10Z. *2*, two stimuli, interval, 0.85σ, stim. 1, 10Z; stim. 2, 56Z. *3*, two stimuli, interval, 0.95σ, stim. 1, 10Z; stim. 2, 56Z. *4*, two stimuli, interval, 2.2σ, stim. 1, 10Z; stim. 2, 17Z. *5*, double response to single powerful stimulus, break shock from coil 2 with iron core, 212Z.

B. January 20, 1922. Temp. about 34°C. Distance stim. to leads, 13 cm.

In records *1* to *4* first stim., 8Z; in records *2* to *4* second stim., 46Z. *1*, first stim. alone. *2*, stim.-interval, 0.85σ. *3*, stim.-interval, 1.0σ. *4*, stim.-interval, 1.2σ. *5*, double response to single powerful stimulus, make shock from coil 2 with iron core, primary current, 0.1 amp., coil distance, 0; no other coil in parallel (corresponding break shock is 630 Z).

In both experiments, 17,000-ohm string, diam., 2.75μ; tension 140 m. per amp. Magnification about 800 (records enlarged from film).

response of nerves. In short, by extending our observations over a wide range of stimulus-intervals and by using a very strong second stimulus, we have shown that this type of correlation is not, as Lucas was led to suppose, peculiar to the more complex case of the response of muscle stimulated through its nerve, but occurs also in the responses of the nerve itself.

Theoretical deduction. Let us now return to the question whether the observed correlation of response-interval with stimulus-interval

is compatible with our proposed explanation of the cause of the delay in the second reponse, i.e., that the second stimulus, falling at a time when the tissue is refractory, cannot at once evoke a response, but does so later when the tissue has recovered, by virtue of the duration of the exciting current and of the resulting local excitatory process. Let us consider what determines whether at any given moment a propagated disturbance may be initiated or not. A survey of the work of Nernst (27), Hill (28) and Lucas (11) suggests that there are two variable factors, one, the intensity of the local excitatory process and the other, the ease with which the propagated disturbance may be set up. During the absolute refractory phase a new response cannot be set up; during the relative refractory phase the local excitatory process must be made more intense than at other times in order to initiate a propagated disturbance. We are accustomed to measure the threshold of excitation in terms of the strength of induction shock required to excite. But since the stimulating value of an exciting current depends on its duration as well as on its intensity it would seem that this usual criterion is at best an indirect one. It seems to us that the true measure of excitability is the intensity of the local excitatory process requisite to excite. We may then conceive of the relative refractory phase as a time during which the local excitatory process must be more intense than usual in order to excite, and during which the requisite intensity gradually returns to its normal value. We have no means of plotting the curve of the intensity of the local excitatory process set up by an induction shock. It is difficult enough to plot accurately the curve of intensity of an induction shock itself (cf. 10). The momentary intensity of the local excitatory process is probably a complex function of the strength and duration of the exciting current which causes it, but we may safely suppose that it lags slightly behind the current in its time relations. Judging from the quantitative data of Lucas (12), the local excitatory process probably only slightly outlasts the induction shock. Indeed, when we consider the brevity of the summation intervals reported by Lucas, and compare them with the figures given by Erlanger and Garrey (10) for the duration of induction shocks derived from ordinary coils, we might even be led to suspect that the summation of inadequate stimuli in the cases studied by Lucas depended on the duration of the shocks themselves, and did not necessarily involve the persistence of any other process after the exciting current had ceased to flow. On the other hand, the well-known fact that with a constant current of brief duration both the intensity and the duration determine whether it shall excite or not, and the fact that alternating currents of high frequency and great intensity may pass through the tissues without exciting them, warrant

the conclusion that the local excitatory process is something distinct from the exciting current itself, and does appreciably outlast it. Although we have no means of ascertaining the exact shape of the curve correlating the intensity of the local excitatory process with time, we may safely assume that its shape will not differ much from that of the induction shock which causes it.

Again, we have no way of knowing the exact shape of the recovery curve of excitability in terms of the intensity of the local excitatory process requisite to initiate a propagated disturbance; but we shall probably not be far wrong if we assume that it is of the same general

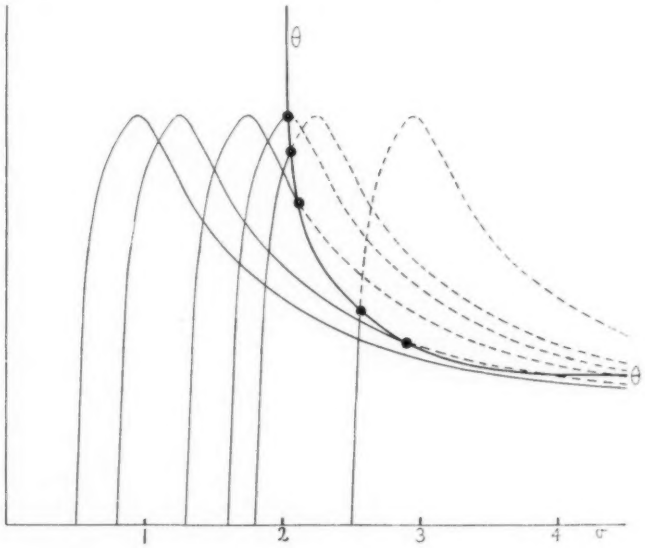


Fig. 9. Theoretical curves to illustrate possible explanation of correlation between stimulus-interval and response-interval. θ-θ, assumed recovery curve of threshold following first response. Abscissae, time in σ. Ordinates, intensity of local excitatory process (see text).

type as the recovery curves that have already been plotted in terms of the strength of current required to excite (6), (9). We may take it for granted that as soon as the local excitatory process attains the value necessary to initiate a propagated disturbance it will do so; let us further assume that the local excitatory process resulting from an induction shock rises to maximum and falls away again according to a curve similar in shape to that which has been established for the induction shock itself; and let us also assume that the intensity of this process requisite for excitaton falls gradually to normal in the same

manner as the empirically measured threshold of excitation. Let us see whether the mutual relations of such curves could possibly account for such a correlation between response-interval and stimulus-interval as we have observed. In figure 9 we have plotted a hypothetical recovery curve of threshold, in terms of local excitatory process. On the same graph we have plotted a series of curves, each representing the course of the local excitatory process resulting from an induction shock and each at a different interval after the first stimulus. In each case, the point where the curve of the local excitatory process resulting from the shock meets the curve of the threshold, indicated by round dots, will be the moment at which the second response will begin. The times of intersection have been purposely chosen to represent approximately the observed times of second response in the experiment whose data are plotted in figure 6A. The significant point is that the shapes of the hypothetical curves which we have chosen to represent the course of the actual excitatory process and of the recovering threshold in order to make the theoretical correlation agree with the facts, are not at all improbable. The significant features are as follows: a gradual disappearance of the delay represented by the curved portion of the graph in figure 1B depends on the fact that the local excitatory process must take an appreciable time to attain its maximum value, and the higher the threshold, the longer will be the time required to reach it; the bending back of the curve (fig. 1C), representing an actual increase of the interval between responses at the shortest stimulus-intervals, must depend, by this hypothesis, on the possibility that the threshold at some time declines more rapidly than the local excitatory process subsides after the decline of the induction shock, so that the recovery curve overtakes the stimulus curve.

It is conceivable that this latter condition might be found only with a certain combination of temperature and duration of induction shocks. It is possible, for instance, that only with an induction coil producing break shocks of considerable duration could this type of curve be obtained. In this connection it is interesting to note the difference in deformation by powerful shocks in action current records from nerves stimulated with coils 1 and 2, already published in a previous paper (15). Coil 1 was designated "Coil X" in that paper, and coil 2 was called "Coil F". We were unable to determine with certainty the cause of these deformations; the only explanations we could offer were an electrical artefact ("escape of stimulating current") and double or multiple response in the nerve. The complex deformation with the strongest shocks is probably due to a combination of these causes. The deformation caused by coil 2 with its fixed iron core was far greater than that caused by coil 1 without its core (as we have used it in most

of these experiments), and this suggests a long duration of the shocks delivered by coil 2, favorable to the production of the effect shown by the bending back of the curve in figure 6. On this hypothesis, with the type of coil he used, Gotch might still have been unable to obtain this bending back of the curve, even if he had used extremely powerful stimuli at briefer intervals than those at which he first obtained second responses. The failure of the sartorius muscle in Lucas' experiments to show this type of curve may have depended on the brevity of the induction shocks and their rapid decline as compared with the comparatively gradual recovery of threshold in muscle fibers. It would be absurd to suppose that the curves as drawn could be quantitatively accurate, since we do not know the nature of the quantities which we are plotting. They are merely intended to show a reasonable basis for interpreting the type of relation which exists between stimulus-interval and response-interval. This hypothesis, however, seems to be entirely in harmony with the facts of our own experiments and also with those of Lucas and Gotch.

TABLE 1

STRENGTH OF STIMULUS	RESPONSE INTERVALS IN σ						AVERAGE
	Beginning			Peak			
	F.	R.	G.	F.	R.	G.	
39 Z	6.3	6.5	6.3	6.4	6.5	6.3	6.4
56 Z	6.2	6.1	6.1	6.0	6.1	5.9	6.1
68 Z	5.7	5.6	5.8	5.9	6.0	6.0	5.8+
81 Z	5.8	5.7	5.7	5.8	6.0	5.7	5.8−

In this connection it is of interest to note that when a single powerful induction shock has evoked two separate responses in a nerve, the response-interval has proved to be approximately the same as that obtained with two stimuli at the briefest interval. Examples of this are shown in figure 8, in which this observation appears in each of the two experiments illustrated; the response-intervals are, as nearly as we can measure them, 2.1σ (A5) and 1.8σ (B5).

If our hypothesis is correct it should be possible at the briefer stimulus-intervals to reduce the response-interval by increasing the strength of the second stimulus, for in this way the local excitatory process would be made to rise more rapidly and the curve representing its intensity would then intersect the curve of threshold at an earlier time. In a few of the experiments with both frog and cat nerve we have varied the strength of the second stimulus at some of the earliest intervals at which it could be made effective. The possible change in latency of the second response in such an experiment would

at best be small. We could scarcely hope that it would greatly exceed the possible error in observation, especially since the early second response would be small, and both the time of onset and of the peak would be difficult to determine accurately. Nevertheless we have obtained one experiment with a frog nerve at 15°C. in which there is little doubt that at the earliest stimulus-intervals the latency of the second response shows a real decrease as the strength of the second stimulus is increased. In table 1 are given the measurements upon which this statement is based, as made independently by each of us. In each case the interval between stimuli was 5.0σ. In the first column is shown the strength of second stimulus in Z units, and in the remaining columns are shown separately the measurements from the beginnings of the responses and from their peaks. In the final column is the general average of all measurements from each observation. The agreement in our measurements seems to indicate that the difference in latency resulting from change in the strength of the second stimulus is real, being too large to be explained by observational error. In figure 10A are reproduced the records obtained in this experiment with two widely different strengths of second stimulus.

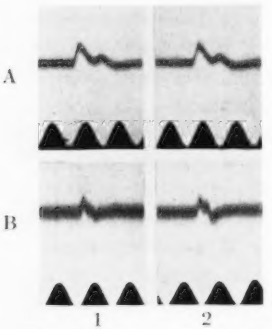


Fig. 10. Records showing slight decrease in response-interval on increasing the strength of second stimulus with stim.-interval unchanged.

A. May 25, 1923. Bullfrog sciatic nerve. Temp., 15.6°C. Distance stim. to prox. lead, 31 mm.; distance between leads, 9 mm. Stimulus-interval, 5.0σ. First stim., 43Z in both. Second stim., in *1*, 39Z; in *2*, 68Z. 16,600-ohm string, diam., 1.25μ; tension, 102 m. per amp.

B. November 21, 1921. Cat peroneal nerve. Stimulus-interval, 1.3σ. First stim., 52Z in both. Second stim., in *1*, 56Z; in *2*, 68Z. Temp., in *1*, 32.6°C.; in *2*, 31.6°C. 16,000-ohm string, diam., 1.5μ; tension, 98 m. per amp. Records enlarged from film; magnification about 800.

A mammalian nerve at 21.5°C. showed a similar result. With a stimulus-interval of 2.0σ a second stimulus of 31.5 Z evoked a second response after an interval of 3.4σ; when the strength of the second stimulus was increased to 38 Z the interval was reduced to 3.0σ. In a mammalian nerve at body temperature similar variation in the strength of the second stimulus seems to produce a similar result. Here the possible error of observation is relatively so large as to render the results more doubtful, but the average of measurements tends to support the same conclusion. The data in one mammalian experiment are as follows: temperature 32°C., interval between stimuli 1.3σ. From two separate observations with a

second stimulus of 56 Z units the measurements for the interval between the responses were 2.1σ and 2.0σ. From two observations taken immediately afterwards with a second stimulus of 68 Z units the measurements of the response-intervals were 1.9σ and 1.8σ. One observation at each strength of the second stimulus is reproduced in figure 10B. With the stronger stimulus the second response appears larger, which probably means that the weaker second stimulus failed to excite all the fibers.

In two other mammalian experiments the results appear at first sight to be contradictory to the conclusion indicated by the experiments just cited. But examination of the mechanical contractions, simultaneously recorded, reveals a possible explanation of the apparent contradiction. The appearance of summated contraction when the strength of the second stimulus was increased in these experiments can be explained as due to the excitation of additional nerve fibers with a longer refractory phase than those that responded only to the weaker stimulus, quite as easily as on the ground of delayed time of response in the individual fibers. Either explanation would harmonize with the apparent increase in the response-interval, and the former seems on the whole more reasonable since it does not conflict with other observations.

As already mentioned, these mammalian records at body temperature involve such brief time intervals that the measurements are at best of doubtful value. We must therefore place our reliance on the experiment on the frog nerve at 15°C. in which the intervals are greater (table 1). This experiment was carefully controlled, and there seems to be little doubt here that increasing the strength of the second stimulus did in reality decrease appreciably the response-interval, although the stimulus-interval remained unchanged. As far as the evidence goes it tends to support the general hypothesis we have proposed, i.e., that the time of second response in the nerve depends upon the moment at which the local excitatory process set up by the second stimulus reaches the requisite intensity for initiating a propagated disturbance, this requisite intensity, or threshold, returning gradually to normal as the tissue recovers from the previous response. The chief advantage of this hypothesis lies in the fact that it involves no radically new assumptions; it simply depends on the correlation of facts and principles already fairly well established.

Diminished velocity of conduction in nerve during the relative refractory phase. We have already mentioned Gotch's suggestion that the second response is delayed because it is propagated along the nerve more slowly than the first. We also mentioned Lucas' objection to this view, in which he argued that it would only serve to

explain the delay of the second response to a fixed interval after the first, but would fail to explain the type of correlation actually found and described above. This argument needs further analysis. It is undoubtedly true that if during the relative refractory phase conduction is slower than normal, the second response must ultimately lag behind the first until it drops back into a portion of the fiber so completely recovered as to conduct at normal velocity. Whether the result of slowed conduction would be a fixed time for the response to an early second stimulus, as Lucas maintained, or the type of correlation found in Gotch's experiments (fig. 1B), would depend on where the responses were recorded; if far enough away from the point of stimulation, the second responses would all appear at the fixed interval after the first, postulated by Lucas; if recorded near enough to the

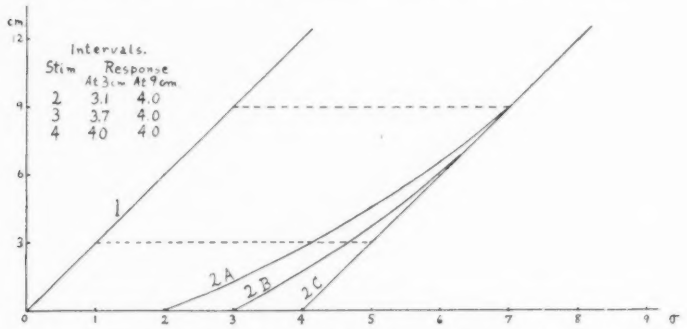


Fig. 11. Theoretical curves to show the lagging of an early second nerve impulse behind the first, according to the hypothesis of Gotch. Abscissae, time; ordinates, distance along nerve. Curve *1* is the course of the first impulse. Curves *2a*, *2b* and *2c* represent the second impulse set up at various times after the first. (See text.)

point of stimulation the second response could be found while still falling behind the first response, but not yet far enough behind to be travelling at the same velocity. This point is illustrated in figure 11 in which distance along the nerve from the point of stimulation is plotted against time. The course of the first impulse is shown by the straight sloping line, the course of the second impulse at two different stimulus-intervals, according to Gotch's hypothesis, is shown by the two curved lines. The intervals between stimuli are shown by the points at which the curves leave the base line. The intervals between responses which would be found near the point of stimulation, on this view, are shown by the intersections of the lower horizontal broken line with the curves. The constant interval between responses, which would be found if they were recorded far enough away from the point

of stimulation, is shown by the intersections of the upper broken line with the sloping lines representing the first and second impulses respectively. In this figure the velocity of the impulse is expressed by the slope of the curve. The first impulse, having presumably constant velocity, is shown by a straight line. The curve expressing the progressively changing velocity of the second impulse with a stimulus-interval of 2σ (curve 2A) is arbitrarily drawn to illustrate the application of Gotch's hypothesis, and it is assumed for the purpose of the figure that the velocity of the first impulse is 30 m. per second, and that 4σ is the shortest time thereafter at which conduction at the same velocity can again occur. The curve for the second impulse with a stimulus interval of 3σ (curve 2B) is derived from curve 2A on the assumption that the velocity of the impulse at any point depends on the interval at which it follows the first impulse at that point. This is done by making the slope the same as that of curve 2A at the same horizontal distance from curve 1. The inset in the figure shows in tabular form how the response-interval would, on this hypothesis, depend not only on the stimulus-interval but also on the point where the responses were recorded.

It would seem then that Lucas' objection to Gotch's suggestion is not necessarily valid. The question should be investigated by experiment. With the ordinary laboratory frog, however, the longest nerve is so short that it is not easy to get even one pair of leads far enough away from the stimulating electrodes to avoid possible confusion in measuring the time of the second response due to the electrical artefact involved in applying the strong second stimulus needed to excite the nerve during its relative refractory phase. It would be extremely difficult, if not impossible, to apply to an ordinary frog's nerve proximal and distal pairs of leads far enough apart to demonstrate any difference in the speed of conduction, and at the same time to have the proximal pair far enough away from the stimulating electrodes to avoid confusion from the artefact. For this reason we have conducted most of our experiments on mammalian nerves, supplemented in the case of two experiments by nerves from large bullfrogs. Most of our experiments on the point at issue have been performed on the cat's popliteal-tibial nerve (about 20 cm. long) dissected as described in an earlier paper (25). A few have been performed on the peroneal nerve which, though not as long, is much longer than can be obtained from a frog, and has the advantages of being easy to dissect, uniform in caliber, and free from branches over its entire length of 12 to 13 cm. We placed the nerve in a moist chamber, usually at room temperature, with the stimulating electrodes far enough away from the cut end to secure an approximately normal threshold of stimulation (15, p. 179),

and with the proximal pair of leads about 10 or 12 mm. apart and from 15 to 30 mm. distant from the stimulating electrodes, this being about as near as they can be placed without fear of confusion from the electrical artefact. The distal pair of leads was placed as far from the proximal leads as possible without rendering the responses monophasic. The two pairs of leads were connected with a double-pole double-throw switch whereby either pair could be connected with the galvanometer. The nerve was stimulated with the Lucas pendulum arranged to apply two stimuli, as already described. The absolute velocity of the nerve impulses was not measured; that is, no effort was made to determine the time between stimulus and response. The question of slowed conduction was tested merely by comparing the interval between the two responses at the proximal and at the distal pair of leads.

In eight experiments on mammalian nerves and in two on bullfrog nerves we have obtained satisfactory results with this method. A difficulty in making measurements in these experiments arises from differences in velocity of conduction between individual fibers. The resulting dispersion in the time when individual impulses arrive at a distance from the point of stimulation has been noted in a previous paper (25, p. 455), and more accurately examined and analyzed with a Braun tube by Gasser and Erlanger (17). This dispersion makes the record less abrupt and sharply defined. With an early second response, which is subnormal, the dispersion makes especially difficult a determination of the exact point in the record where the impulse first appears. The measurement will tend to indicate a greater delay at the distal leads than actually occurs. We have tried to detect the very first divergence between the records of single and double stimulation, in order to minimize this source of error. In most cases several tests were made at each stimulus-interval, and each record was measured independently by all three, or at least two of us. Table 2 shows the averages of these measurements in the mammalian experiments. In the third column is given the distance in millimeters from the point of stimulation (kathode on break shock) to the proximal lead of the proximal pair: in the fourth column is the distance from the proximal lead of the proximal pair to the proximal lead of the distal pair. The leads of each pair being only 10 to 12 mm. apart, the distance separating the distal leads of the two pairs was in each case about the same as that given in the table for the proximal leads. In the next three columns are the intervals between the stimuli and between the responses recorded at the two pairs of leads. In the final column is the percentage increase in response-interval at the distal pair of leads over that found at the proximal pair. In figure 12 records are shown from a typical experiment in this series.

In our earlier experiments the increase in response-interval appearing even in so great a distance as 10 cm. along the nerve, seemed to be so small that we suspected that our measurements were in error on account of the dispersion referred to above, and that there was in reality

TABLE 2

DATE	TEMPERATURE	DISTANCE, MM.		STIMULUS INTERVAL	RESPONSE INTERVAL		PERCENT INCREASE
		STIMULUS-P_1	P_1-P_2		At proximal leads	At distal leads	
	°C.						
Nov. 8, 1922	21.5	26	60	2.0	3.4	3.5	3
				2.0*	3.0	3.8	27
		27	43	1.6	3.1	3.1	0
				2.0	3.3	3.3	0
Nov. 15, 1922	22.4	21	63	2.3	3.6	3.5	−3
Dec. 12, 1922	21.8	15	102	1.9	3.5	3.7	6
				2.2	3.4	4.0	18
Apr. 12, 1923	22	17	82	2.2	2.9	3.9	34
				2.45	2.9	4.3	48
				2.75	3.2	4.4	37
May 3, 1923	23	24	91	2.2	3.0	3.8	27
				2.3	3.0	3.3	10
				2.45	3.9	3.8	26
				2.75	3.1	3.7	19
May 8, 1923	26	22	98	2.5	3.1	3.3	6
				3.0	3.4	3.5	3
				4.0	4.3	4.1	−5
May 15, 1923	20	15	130	2.5	3.1	4.3	39
				3.0	3.5	4.5	29
				3.5	3.9	5.0	28
				4.0	4.3	5.0	16
				5.0	5.3	5.7	8
				6.0	6.4	6.4	0

* Stronger second stimulus.

no slowing of conduction of the second impulse. But a conference with Doctors Gasser and Erlanger convinced us that a nerve impulse set up in the relative refactory period is actually retarded in transmission. They have, with their more accurate method of recording by means of a Braun tube, investigated this point in connection with another problem, and have kindly permitted us to mention their as

yet unpublished observations. In our later experiments (see table 2) we have found clear evidence that a nerve impulse set up during the relative refractory period is not only of subnormal magnitude, but also travels more slowly than normal, and therefore lags behind a normal impulse which precedes it. The increased interval found in our experiments is about the same as the more accurate determinations of velocity by Doctors Gasser and Erlanger would lead us to expect.

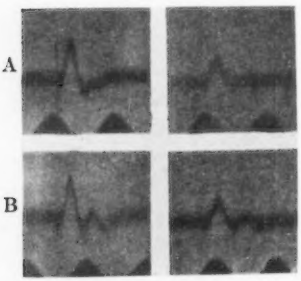


Fig. 12. Records showing reduced velocity of nerve impulse during relative refractory period.

December 19, 1922. Cat peroneal nerve. Temp., 19°C. Proximal pair of leads 12 mm. apart, 22 mm. from stimulus; distal pair of leads 13 mm. apart, 82 mm. from stimulus. High speed camera. 4,800-ohm string, diam., 2μ; tension, 80 m. per amp. Magnification, about 700.

A. First stimulus alone. B. Two stimuli, interval, 2.65σ.

P, recorded at proximal leads; *D*, at distal leads. First stim., 10Z; second stim., 32Z.

In plotting figure 11 we assumed that the closer the second impulse followed the first, the slower would be its velocity. If slowed conduction were a feature of the relative refractory phase we should expect the conduction rate to return to normal gradually, as do the threshold and the size of response, rather than abruptly. Nevertheless it is worth while to examine the data of experiments in which different stimulus-intervals were used, and see what they show concerning the recovery of the normal velocity of conduction. Most of the data in table 2 are not adequate for this purpose, since the difference in stimulus-intervals was not great enough, and at the temperatures employed the observational errors were relatively too large to render possible any clear correlation of the slowing of conduction with the stimulus-interval. Therefore we performed three experiments on cat nerves (two of which are included in table 2) and two on frog nerves, in which we used the procedure described above for detecting slowed conduction, and varied the stimulus-interval over the entire range of the relative refractory period. In one of the mammalian experiments we cooled the nerve below room temperature in order to increase all the intervals, and thus facilitate accurate measurements. Since previous experiments (25) showed that at temperatures below 15°C. the action current of a mammalian nerve becomes impaired, we kept the temperature of this nerve between 16° and 17°C. Normal conduction nevertheless was interfered with, for with all stimulus-intervals up to 8σ the response-interval at the distal leads (120 mm. from the stimulus) was approximately 15σ, although at the proximal leads there

s the usual correlation (see fig. 6C), the delay in the second response practically disappearing with a stimulus-interval of 8σ. We concluded that the mammalian nerve would not conduct normally at so low a temperature, and repeated the experiment at 20.3°C. with the result which appears under date of May 15 in table 2. We also performed the same experiment on two bullfrog nerves at 12.5°C. and 15.3°C. In the former most of the stimulus-intervals proved to be too brief to give a clear second response; we failed therefore to get enough data to show clearly the gradual return to normal of the speed of conduction. In the experiment at 15.3°C., however, this was shown quite satisfactorily. The results are given in table 3. They are also plotted graphically in figure 13 by the method adopted in figure 11. In order to complete the figure it has been necessary to assume the velocity of the first impulse, which our data do not show. This velocity is assumed to be 20 m. per second, a convenient approximation to that indicated by classical researches.

TABLE 3

STIMULUS INTERVALS IN σ	RESPONSE INTERVALS IN σ							
	Proximal leads 31 mm. from stim.				Distal leads 91 mm. from stim.			
	R.	G.	F.	Average	R.	G.	F.	Average
6.0	6.7	6.5	6.9	6.7	6.9	6.9	7.3	7.0
7.0	7.8	7.5	8.2	7.8	8.8	8.6	9.1	8.8
8.0	8.8	8.8	8.9	8.8	9.4	9.5	9.6	9.5
10.0	10.7	10.4	10.6	10.6	10.9	10.9	11.5	11.1
12.0	11.9	11.9	12.7	12.2	12.1	12.3	13.1	12.5

In a general way these results harmonize with the theoretical picture presented in figure 11; only they show that the slowing of conduction is so slight that a much greater length of nerve would be required for the lagging of the second impulse to reach its limit and cease. Thus it appears from quantitative experiments that Lucas' objection to Gotch's hypothesis of slowed conduction is not valid. Our records also indicate that slowed conduction persists about as long after the refractory phase as the subnormal magnitude of response. And the return to normal of the velocity of conduction is gradual, just as is the return of the possible size of response. This is illustrated in figure 14, in which the size of the second response is shown both when conduction was considerably slowed and when its velocity was almost normal. The records are from two separate experiments, one of which furnished the data for figure 13.

There are so many facts pointing to the view that the electric response is the actual mechanism of conduction in nerve and muscle

fibers, that it seems worth while to look for a possible clew to the cause of reduced velocity during the relative refractory period in terms of this theory of conduction. Lillie (29) and Lucas (20) have offered good reason for supposing that the velocity of conduction depends on the rate of development of the electric response at a given point in the fiber; and Lillie found that the distance between electrodes on a nerve trunk at which an impressed difference of potential approximating that

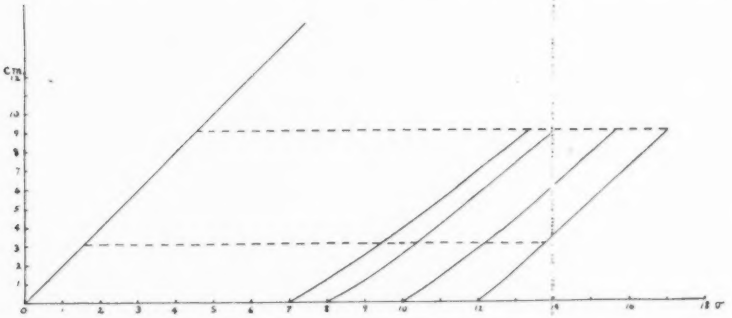


Fig. 13. Curves showing velocity of nerve impulses initiated at intervals from from 7 to 12σ after the first impulse; plotted from the data in table 3 on the assumption of 20 m. per second as velocity of first impulse. Abscissae, time; ordinates, distance along nerve.

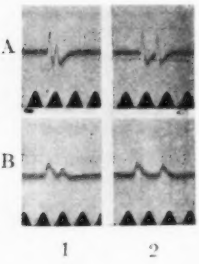


Fig 14. Records from two experiments showing recovery of size of electrical response correlated with return of velocity of conduction to normal after a previous impulse.

A. May 15, 1923. Cat popliteal-tibial nerve. Temp., 20.4°C. Distance stim. to leads, 15 mm. First stim., 28Z; second stim., 35Z. Stimulus-intervals: in *1*, 3.0σ; in *2*, 7.0σ.

B. May 25, 1923. Bullfrog sciatic nerve. Temp., 15.5°C. Distance stim. to leads, 31 mm. First stim., 44Z; second stim., 46Z. Stim.-intervals: in *1*, 6.0σ; in *2*, 12.0σ. In both experiments 16,600-ohm string, diam., 1.25μ; tension, 102 m. per amp. Magnification, 490. For relative velocity of second impulse in each case, see data in tables 2 and 3 and in figure 13.

found in the action current barely sufficed to excite the nerve, was just about enough to account for the propagation of the nerve impulse at the velocity actually found. The probable limitation of the rate of rise of the electric response and of the resulting speed of conduction by the physical constants of the fiber has been discussed in a previous paper (30). According to this view of the mechanism of conduction the velocity will depend on two things,—the rate at which the difference

of potential develops between the point just reached by the impulse and the inactive part of the fiber ahead of it, and the ease with which the inactive part of the fiber can be excited, i.e., the difference of potential required to excite it. Now if the action current does indeed provide the means of conduction in the manner suggested above, then conduction must be slower than normal during the relative refractory phase. In the first place, the action current is smaller than normal at this time, and therefore, if its time relations are unaltered, its rate of rise at a given point must be correspondingly reduced. Only if the peak followed the onset more closely than normal could the rate of rise remain unchanged in the case of a subnormal response. Lucas' capillary electrometer records of both nerve (13) and muscle (2) responses show clearly that this is not so, and that the rate of development of the difference of potential is less in the subnormal than in the full-sized response. In the second place we know that the threshold of excitation is higher than normal during the relative refractory period, and this is probably so whether the stimulus is an externally applied current or the electric response of an adjacent section of the fiber. Therefore, when the action current reaches a given intensity it will not be able in the relative refractory phase to excite the fiber as far away from the active point as at other times. Each factor will contribute to the slowing of the impulse; the reduced rate of development of the electric response and the reduced excitability of the fiber will combine to delay conduction. As the nerve recovers from the refractory phase the size of response which it can yield and the threshold of excitation both return gradually to normal. Therefore, with both causes of delayed conduction gradually disappearing, we should expect the velocity of conduction to return gradually to normal. And this is what our experiments show to be the case. This agreement between prediction and observation may lend additional support to the view that the action current is the essential mechanism of conduction.[3]

[3] There appears to be a contradiction between our conclusion that in the relative refractory period velocity of conduction depends in part on the size of the disturbance and Adrians' conclusion (J. Physiol., 1914, XLVIII, 53) that in a narcotized nerve velocity does not depend on the size of the disturbance. It is premature to attempt to explain away this apparent contradiction, but it should be noted that the mechanism of "conduction with a decrement" in narcosis is still obscure, and that so far as we are aware there is as yet no proof that the electric response becomes progressively smaller in a "region of decrement." It is conceivable that in a region of uniform narcosis the size of the electric response is subnormal, but remains constant if it persists at all, and that the apparent "decrement" may in some way be explained by the increasing difficulty which increasing the length of the narcotized region would impose on the subnormal action current in exciting the normal tissue beyond.

The question now arises whether slowed conduction may not account for the entire picture of delay in the second response. We have already shown that our hypothesis of the persistence of the local excitatory process initiated during the refractory phase would probably account for the observed correlation between stimulus-interval and response-interval as indicated in figures 1C and 6. But since the second impulse travels more slowly than the first, and since Lucas' objection to the explanation of the results by slowed conduction has been cleared away by plotting graphically the consequences of such slowing (fig. 11), may we not explain the entire delay by means of delayed conduction?

A little quantitative consideration may help us here. In the average of our experiments the increase in response-interval over a distance of 80 mm. on the nerve amounted to only about 10 or 20 per cent. Yet according to the theory of slowed conduction, the increase in response-interval from the point of stimulation to the proximal leads—a distance of only about 20 mm.—must have amounted, in some experiments, to more than 100 per cent. This great difference appears at first sight adverse to the theory of slowed conduction as explaining the entire delay of the second response. It might be argued, however, that the greater slowing, when the second stimulus is first effective, would be sufficient to account for the greater relative lag in the first few centimeters. It would be difficult to detect an earlier second response than the earliest we have found, since some of these have appeared as barely perceptible displacements of the galvanometer curve (e.g., fig. 4), and the earlier the second response, the smaller it is. If an earlier second response than the earliest we have found could occur in the first few millimeters traversed, it would be difficult to infer its velocity at this early stage by extrapolation from the measurable data of the later stages. But we see no reason to suppose a second nerve impulse can occur much earlier than the earliest we have found. As we experimentally decrease the interval between responses, the second response grows smaller and its size appears to approach zero so rapidly that we are led to suppose that at a time not much earlier than that of the earliest second responses we have found, no response at all can occur. It seems to us, therefore, much simpler and more in harmony with our observations to conclude that the second response, instead of beginning at what seems to be an impossibly early time and then dropping rapidly behind its predecessor in the first part of the fiber it traverses, merely does not occur at all until the nerve has recovered to the point at which we find it able to respond, and then responds in consequence of the persistent local effect resulting from the exciting current. It should be noted also that the increased response-interval with the shortest stimulus-intervals, shown by the bending back of

the curve in figure 6, cannot be explained on the basis of slowed conduction, while it harmonizes perfectly with the persistence of local excitatory process.

THE DELAY IN TRANSMISSION FROM NERVE TO MUSCLE. We now come to consider the delay in the response of a muscle to the second of two stimuli applied to its motor nerve. We have already noted that Lucas found a delayed appearance of the response to an early second stimulus in this type of experiment, and that the correlation between stimulus-interval and response-interval was of the type shown in figure 1C,—a type of correlation found in some of our experiments on nerve alone. Lucas also noted that when muscle was stimulated through its nerve the delay was relatively greater than when either nerve or muscle was directly stimulated, the response-interval in the case of the earliest effective second stimulus amounting to five or six times the stimulus-interval (2, p. 378).

In some of our earlier mammalian experiments we used the nerve-muscle preparation with leads on both nerve and muscle. The stimulating electrodes and the leads on the nerve were shielded and were covered by the skin which was sewed around the necks of the shielding tubes to maintain an approximately normal body temperature. Other leads were applied to the surface of the innervated muscle through small windows cut in the skin. This was done both with the peroneal nerve and tibialis anticus muscle, and with the popliteal-tibial nerve and the external interosseous muscle, as already described (16). With this arrangement we could observe the responses of either nerve or muscle to stimuli applied to the nerve.

We found that at temperatures slightly below normal (e.g., 33°C.), two stimuli separated by an interval of from 0.8σ to 2.0σ will set up two nerve impulses which appear in the galvanometer record with an interval of about 2σ, as already indicated in figure 7; but when the galvanometer is connected with the muscle the electric responses are separated by an interval of approximately 4σ. There appeared to be two distinct delays, one in the setting up of the second nerve impulse by the second stimulus, and one in the setting up of the second muscle response by the second nerve impulse. We obtained this result in two different mammalian experiments, but in order to get the more accurate measurements which a lower temperature will permit, we made a number of similar experiments with the sciatic-gastrocnemius preparation of the frog. Usually the leads were applied to the distal half of the muscle, and only about 1 cm. apart, in order to render the responses small and brief.

The results of these experiments were essentially the same as in the mammalian preparation. An early second stimulus applied to the nerve

evoked a second muscle response with a greater delay than was found in the second response of the nerve. As a rule the second stimulus was so timed that the nerve should have nearly completed its recovery and that its second response should occur with little or no abnormal delay. In this case only the delay in transmission from nerve to muscle was demonstrated; in other cases with briefer intervals between stimuli both delays were shown.

Experiments revealing the additional delay in frog-muscle response are listed in table 4. The average temperature of the preparation is stated in the second column. In no case did the temperature vary much more than one degree above or below this value. The third

TABLE 4

DATE	TEMPERATURE	INTERVAL BETWEEN STIMULI σ	INTERVAL BETWEEN RESPONSES	
			Nerve σ	Muscle σ
	°C			
May 17, 1922	26	1.0		3.8
		2.0		3.8
		2.8		4.0
		1.1	1.9	
		2.0	2.0	
		2.8	2.9	
May 24, 1922	23	2.3	2.9	3.3
		3.1	3.4	3.6
		3.25	3.2	3.6
June 14, 1922	23	2.65	2.7	4.7
		2.95	2.9	5.2
Feb. 2, 1923	18	3.05	3.1	6.8
		3.75	3.8	6.7

column gives the interval between stimuli, and the fourth and fifth columns the intervals between responses of nerve and muscle respectively.

In all cases except that of May 17, the leads were shifted between nerve and muscle without opening the moist chamber (see p. 559), sometimes twice in the same experiment. In the experiment of May 17, the leads were first placed on the muscle and records taken. Then the corresponding nerve-muscle preparation from the opposite leg of the same animal was set up in exactly the same way, except that the leads were applied to the nerve. The figures show that there is at such intervals as these a marked delay in the setting up of the second muscle response by the second nerve impulse.

Let us now consider the significance of this additional delay appearing in the transmission of the second disturbance from nerve to muscle. In a previous section we have brought out the fact, established both by our experiments and by those of Gasser and Erlanger, that a nerve impulse set up during the relatively refractory phase travels more slowly than normal. The question arises whether this slowed conduction may not be the sole cause of the delayed appearance of the second response in the muscle. A quantitative examination of the available data may give an answer.

Although a nerve impulse started during the relative refractory phase will lag behind the previous impulse, ultimately this process of lagging will place it so far behind that it will no longer travel with subnormal velocity and will therefore lag no farther behind. We have not determined accurately the precise intervals at which the lagging ceases, but we have data showing approximately when this state is reached at two or three different temperatures. The experiment on the bullfrog nerve at 15.3°C. shows only a slight slowing of conduction with a stimulus-interval of 10σ, and almost complete disappearance of slowed conduction when the stimulus-interval was increased to 12σ. The temperature coefficient of this effect seems to be large. In one mammalian nerve at 20°C. the slowing of conduction disappeared when the stimulus-interval was increased to 6σ, the response-interval being the same at both proximal and distal leads, and at each pair practically no greater than the stimulus-interval. In another mammalian nerve at 25.5°C. the same result was found at 4σ, as nearly as we can judge from our measurements. In general the time relations do not seem to differ greatly between mammalian nerves and frog nerves at the same temperature.

We have no records of action currents taken at two separate points on a mammalian nerve at normal body temperature, but in two separate experiments in which the nerve-muscle preparation was tested *in situ*, there was evidence that at this temperature a second nerve impulse will follow its predecessor at an interval of about 2σ without lagging further behind it. In one of these,—a popliteal-tibial nerve with the leads about 12 cm. from the point of stimulation—at a temperature of 33°C., double stimulation with intervals of 1.9σ and 2.2σ evoked responses with intervals, as nearly as we could measure them, exactly the same as the stimulus-intervals. The other experiment showed essentially the same result within the limits of observational error. In short, it is quite evident that if a second nerve impulse has dropped back to a certain time interval behind the first (approximately 2σ), it will not lag any farther than this as long as it continues to travel in nerve fibers at body temperature. From this it is clear that the much

greater interval between muscle responses observed in our experiments with mammalian nerve-muscle preparations, cannot have depended on retarded conduction in the nerve. An additional delay must have occurred at some point in the transmission of the disturbance from nerve to muscle.

In the writings of Lucas (31) and Adrian (6), (32) on transmission from nerve to muscle there seems to be a tendency to look on the nerve-muscle preparation as a continuous conducting path,—not homogeneous, but still continuous,—and to identify in the muscle response the same propagated disturbance as the nerve impulse which evokes it. Perhaps it would be better to regard the nerve impulse, or the resulting disturbance at the junction, as the stimulus for the muscle fiber, which responds as a separate physiological unit. The distinction may seem pedantic, but it offers a simple explanation of the additional delay in the appearance of the second response in muscle, on the same principle that we have invoked to explain the delay in the second response in nerve. If the time of the second response of muscle simply depends on recovery from its refractory phase, we need only assume that the nerve impulse, or its effect at the junction, is able to excite the muscle not merely at the moment when it arrives, but for an appreciable time thereafter. Just as we have supposed that the second local excitatory process, though set up when the tissue cannot respond, may outlast the refractory phase and initiate a response when recovery is sufficient, so in the transmission from nerve to muscle we may suppose that the nerve impulse, arriving at the junction when the muscle is refractory, may, either by its own duration or by the disturbance it sets up in the junctional tissue, maintain a source of stimulation until the muscle has recovered sufficiently to respond.

To decide whether the time of appearance of a second response in muscle depends simply on the duration of the muscle's refractory phase, or on some other unknown modification of the conducting path associated with the passage of a previous propagated disturbance and causing at various points along the path varying amounts of delay in the appearance of the second response, we have sought evidence in experiments with three stimuli. If the nerve-muscle preparation were a continuous conducting path, and the muscle response were the same propagated disturbance as the nerve impulse, and if the delay of the second response were due to a special retarding modification resulting from the passage of a previous disturbance, we might then expect the delay in transmission from the stimulating electrodes to the leads on the muscle to be less after a subnormal disturbance than after one of the normal magnitude occurring in a tissue not previously excited. The disturbance occurring in the relative refractory period

following a previous disturbance, is subnormal; therefore a third disturbance set up at the same interval after the second as the second was set up after the first, should find the tissue at least as fully recovered from the second (subnormal) disturbance as the second found the tissue recovered from the first. Probably at a given time after the subnormal response the recovery would be more complete than at the same time after the full-sized response. That this is so in the case of nerve is indicated by the electrometer records of Lucas (13) in which responses to three successive stimuli at equal intervals were carefully analyzed. In one record (fig. 19) the second and third responses appear of practically equal size; in another (fig. 21) the third response is clearly bigger than the second, denoting more complete recovery after the subnormal response than at the same time after the full-sized response. Therefore, when three successive disturbances traverse the nerve-muscle preparation, if the conducting path as a whole is more fully recovered after the second than after the first, we might expect the time elapsing between stimulus and response to be less in the case of the third disturbance than in the case of the second. If, on the other hand, the time of the third response in the muscle fibers depends simply on their refractory period, we should expect to find approximately the same response-interval between second and third responses as between first and second responses. And if the three responses were evoked by nerve impulses at equal intervals so brief that the muscle was unable to respond to the second without abnormal latency, the delay in recovering from a second refractory phase in muscle fibers would necessitate a greater interval between stimulus and response with the third stimulus than with the second.

To test this question we arranged a frog's nerve-muscle preparation in the moist chamber so that the contraction would be recorded, and applied stimulating electrodes to the nerve and leading-off electrodes to the muscle. We connected the secondary coils of three inductoriums in parallel with the stimulating electrodes, and arranged the primary circuits to be broken by the three keys of the pendulum. It is scarcely conceivable that all the fibers in a muscle should have precisely the same refractory period. As a result of slight inequalities we cannot expect all to react just alike to three stimuli. We should expect those with the shortest refractory phase to respond most easily to all three. In a border-line experiment the next most rapid fibers might respond to the first two stimuli, but not to the third. Still slower fibers might respond only to the first and third. We fixed the stimulus intervals so that all or nearly all of the muscle fibers responded to both the first and second stimuli, and surely some of them responded to the third; at the same time we made the intervals as short as pos-

sible while still fulfilling these conditions. The decision as to when the conditions were fulfilled rested chiefly on the height of the recorded contraction. If the first stimulus is maximal, i.e., excites all fibers in the muscle, and then if two stimuli cause a higher contraction, it implies a second response in at least some fibers (8). When at a given stimulus-interval the strength of the second stimulus has been increased till further increase produces no greater summated contraction, we may infer that all the fibers which can respond to a nerve impulse at this time have so responded. We then have a maximal first stimulus and a maximal summation evoked by the second. The greater the interval between the first and second stimuli, the greater will be the summated contraction. This may be due to two causes: *a*, the second muscle response occurring later in the relative refractory phase will be larger and will therefore contribute more to the total contraction; *b*, the duration of the refractory phase is probably not the same in all muscle fibers (an early second nerve impulse may be able to evoke a second response in some muscle fibers, but not in all, whereas a somewhat later second nerve impulse will excite more fibers, and thus increase the total contraction). In applying three stimuli we might find confusion if a considerable number of muscle fibers failed to respond to the second stimulus and therefore responded only to the first and third. To avoid this source of confusion it was necessary to apply the second stimulus late enough to insure a second response in most if not all muscle fibers. This was done by adjusting the stimulus-intervals so that the contraction evoked by all three stimuli was greater than that evoked by the first and third stimuli alone. Even if the second stimulus is so timed as to evoke a second response in all fibers, the amount of contraction evoked by stimuli 1 and 3 will be greater than that evoked by stimuli 1 and 2, because of the greater response in the later stages of recovery. To prove that some fibers have responded three times, it is not enough that the contraction evoked by the stimuli 1, 2 and 3 be greater than that evoked by stimuli 1 and 2. The increased contraction resulting from the addition of the third stimulus may be because some fibers, failing to respond to stimulus 2, respond to stimulus 3. If all three stimuli evoke a smaller contraction than stimuli 1 and 3, the effect of introducing stimulus 2 may be to make fibers with the shortest refractory phase respond earlier than if only stimuli 1 and 3 had been applied. In this case none of the fibers might respond to all three stimuli. Thus if the contraction evoked by all three stimuli is intermediate in size between that of 1 and 2 and that of 1 and 3, we are left in doubt as to whether any of the muscle fibers have responded three times. Only if the contraction to all three stimuli is the biggest of all, can we be sure that some muscle fibers

have responded three times. We have, therefore, in these experiments, varied our stimulus-intervals until we found values at which the record fulfilled this requirement (fig. 16).

In figure 15 we have reproduced significant galvanometer records from three separate experiments performed under the above conditions. Figure 16 shows the drum record in one of the cases, proving the existence of three responses in some of the fibers. In table 5 meas-

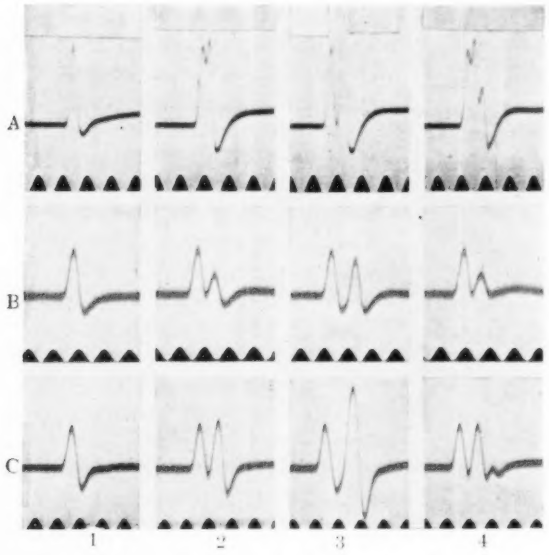


Fig. 15. Records of muscle action currents from three experiments on frog nerve-muscle preparation. In each experiment the setting of the pendulum for the stimulus-intervals was kept constant throughout. The first three records in each experiment are controls; *1* is stimulus 1 alone; *2*, stim. 1 and 2; *3*, stim. 1 and 3; *4*, stim. 1, 2, and 3.

A. May 17, 1922. Temp., 25.5°C. Stim.-intervals: 1-2, 2.65σ; 2-3, 2.65σ. Stim. 1, 18Z; stim. 2, 9Z; stim. 3, 16Z. 20,000-ohm string, diam., 1.75μ; tension, 82 m. per amp.; damped with 0.1 microfarad condenser. Magnification, 350.

B. February 14, 1923. Temp., 21°C. Stim.-intervals: 1-2, 5.0σ; 2-3, 5.0σ. Stim. 1, 4.2Z; stim. 2, 10.5Z; stim. 3, 24Z. 4,800-ohm string; diam., 2μ; tension, 80 m. per amp.; damped with 0.3 microfarad condenser; 3,000-ohm shunt across string to reduce excursions. Magnification, 350.

C. February 21, 1923. Temp., 20.5°C. Stim.-intervals: 1-2, 5.5σ; 2-3, 5.5σ. Stim. 1, 7Z; stim. 2, 9Z; stim. 3, 12Z (approx.). 4,800-ohm string; diam., 2μ; tension, 80 m. per amp. Magnification, 350.

Note: In A the responses to stim. 2, though attaining a greater absolute height than the first response, are probably no larger in reality, for they start from a higher level.

urements showing the results of the experiments are given. The figures are in some cases the measurements made on the observations reproduced in figure 15 and on other similar observations; in others they are averages from observations shown in the figure and from other duplicate observations made immediately before or after these under precisely the same conditions. These measurements are relatively more accurate than most of those on nerve responses, because of the longer time elapsing between muscle responses. The measurements were usually made from the peaks of responses, as these seemed to give more uniform and accurate results than their beginnings. The temperature given for each experiment is the approximate average, the deviation during the experiment not amounting to more than about 1°C. Each horizontal line in the table represents a single observation; in each group of four the observations were made in rapid sequence (the entire series being completed in about one minute), and the stimulus-intervals remained unaltered. Each group includes control observations in which two of the three stimuli were applied—the figures in the third column show the stimuli applied. The stimulus-intervals and the corresponding response-intervals are placed side by side. In the last line of each group is the crucial test with all three stimuli. Obviously in the case of three responses the interval between responses 1 and 3 is the sum of the other two response-intervals. In the last two columns are the excess latencies of the second and third responses as compared with the first; i.e., the amount of abnormal delay traceable to the previous response or responses.

Fig 16. Myograph record of the experiment from which the galvanometer records in figure 15A were taken, showing height of contraction with all combinations of stimuli. The combinations were as follows, in the order in which the contractions appear: 1; 2; 3; 1 and 2; 2 and 3; 1 and 3; 1, 2, and 3; 1. The response to each of the single stimuli was maximal; the slight differences between them are due to Treppe.

It is clear that in every case of three responses the interval between stimulus and response became progressively greater from the first to the third.

In two of the experiments represented in figure 15, when stimuli 1 and 3 were applied without stimulus 2, the second electric response was larger than the first, although stimulus 1 was maximal. This appears to exemplify the supernormal phase of recovery described by Adrian

TABLE 5

DATE	TEMPERATURE	STIMULUS USED	INTERVALS IN σ						EXCESS LATENCY OVER 1	
			1-2		2-3		1-3			
			Stimulus	Response	Stimulus	Response	Stimulus	Response	2	3
	°C.									
		1,2	2.65	3.7					1.0	
		2,3			2.65	3.7				1.0
		1,3					5.3	5.7		0.4
		1,2,3	2.65	3.7	2.65	3.7	5.3	7.4	1.0	2.1
May 17, 1922	26									
		1,2	2.8	3.9					1.1	
		2,3			3.05	3.9				0.8
		1,3					5.85	5.8		0
		1,2,3	2.8	4.0	3.05	3.8	5.85	7.8	1.2	2.0
		1,2	4.6	6.5					1.9	
		2,3			4.5	5.7				1.2
		1,3					9.1	9.1		0
		1,2,3	4.6	6.3	4.5	6.0	9.1	12.3	1.7	3.2
		1,2	5.0	7.0					2.0	
		2,3			5.0	6.6				1.6
		1,3					10.0	10.0		0
		1,2,3	5.0	7.0	5.0	6.7	10.0	13.7	2.0	3.7
Feb. 14, 1923	21									
		1,2	5.3	7.0					1.7	
		2,3			5.25	6.3				1.0
		1,3					10.55	10.8		0.2
		1,2,3	5.3	7.1	5.25	6.6	10.55	13.7	1.8	3.1
		1,2	6.0	7.4					1.4	
		2,3			6.05	7.0				1.0
		1,3					12.05	12.0		0
		1,2,3	6.0	7.4	6.05	6.6	12.05	14.0	1.4	2.0
		1,2	5.5	7.8					2.3	
		2,3			5.5					
Feb. 21, 1923	20.5	1,3					11.0	11.5		0.5
		1,2,3	5.5	7.8	5.5	7.5	11.0	15.3	2.3	4.3
		1,2	5.0	6.9					1.9	
Apr. 3, 1923	19.5	2,3			5.0	6.5				1.5
		1,3					10.0	10.3		0.3

TABLE 5—*Concluded*

DATE	TEMPERATURE	STIMULUS USED	INTERVALS IN σ						EXCESS LATENCY OVER 1	
			1-2		2-3		1-3			
			Stimulus	Response	Stimulus	Response	Stimulus	Response	2	3
	°C.									
Apr. 3, 1923	19.5	1,2,3	5.0	6.7	5.0	6.6	10.0	13.3	1.7	3.3
		1,2	5.5	7.3					1.8	
		2,3			5.5	7.0				1.5
		1,3					11.0	11.3		0.3
		1,2,3	5.5	7.2	5.5	6.5	11.0	13.7	1.7	2.7
Feb. 19, 1923	18.5	1,2	8.5	10.9					2.4	
		2,3			8.5	10.5				2.0
		1,3					17.0	17.9		0.9
		1,2,3	8.5	10.7	8.5	10.2	17.0	20.9	2.2	3.9

and Lucas (6), and later shown by Adrian to be dependent on acidity (33). In one experiment this condition only occurred after a good many tests had already been made. In that case the first response became smaller with repetition, and the second response, although bigger than the first (in the case of stimuli 1 and 3) never was bigger than the first had been when the preparation was fresh. Apparently this type of behavior was the result of fatigue; and fatigue is associated with acidity (33).

The question arises whether this condition vitiates the experiment as a basis for conclusions concerning delay. A properly timed second response larger than the first might represent an actually greater action current in the individual muscle fiber, because occurring in the supernormal phase of recovery; but Adrian has shown (9) that in cardiac muscle, and probably also in nerve, the *electric* response in the supernormal phase is no larger than the first response. It is unlikely that skeletal muscle differs in this respect from both cardiac muscle and nerve.

The only reasonable interpretation of the supernormal phase of recovery, observed by us, seems to be along lines indicated by Adrian and Lucas (6). On this view fatigue causes a block at the neuromuscular junction. This block is sufficient at some of the junctions to extinguish the first propagated disturbance, so that the first stimulus does not excite all the muscle fibers; hence the total response is smaller than when the preparation was fresh. The second nerve impulse,

when properly timed (as in the case of stimulus 3), arrives at the junction during the supernormal phase of recovery, and where formerly blocked, is able to get through and excite fibers unaffected by the first stimulus.

Could this condition in any way interfere with our conclusion as to a progressive increase in delay in the experiments with three stimuli? The failure of some of the muscle fibers to respond to the first stimulus because of a block at the junction could only cause confusion if these same fibers responded to the second or third stimulus earlier than if they had already responded to the first stimulus. The case in which the supernormal phase appears—the response to stimulus 3 after 1—was only a control observation, with little or no abnormal delay in the second response. The crucial test was that in which all three stimuli were applied, and in that case there was no certain evidence of the supernormal phase in any experiment; the stimulus-intervals were probably so short that each impulse, after the first, arrived at the junction before the supernormal phase had begun. Probably, therefore, no muscle fibers which had failed to respond to the first stimulus responded to either the second or third. Even if some did, the effect could hardly be to simulate the progressive increase in delay which occurred. We should rather expect them, by responding more promptly, to make either of the later responses as a whole appear to begin earlier.

We may safely conclude, then, that the delay in the second and third responses in our experiments was actual, and that the latency increases progressively from the first to the third response.

In some experiments with three stimuli we temporarily transferred the leads from the muscle to the nerve and determined the response-intervals in the nerve. At stimulus-intervals giving the typical picture of muscle delay shown in figure 15 and in table 5, we found no delay at all in the second or third nerve impulse; that is, no increase in response-interval over stimulus-interval. The progressive increase in the delay of the muscle response therefore occurs entirely in transmission from nerve to muscle.

The response-intervals in these experiments are such that we can more easily interpret them as due to the refractory phase of the muscle than to an effect on the conducting path as a whole, commensurate with the size of the disturbance which has just passed. For, although the first muscle response was full-sized and the second was subnormal, the transmission time of the disturbance which followed the second was always greater than that which followed the first. On the other hand, the interval between the second and third responses was always nearly the same as that between the first and second, but slightly shorter. This harmonizes perfectly with the view that the

refractory phase of the muscle fibers chiefly determines the time of each response after the first, for, as we have already intimated, we should naturally expect the refractory period following a subnormal response to be less than that following one of normal size, and the electrometer records of Lucas seem to show this to be the case in the responses of nerve. In our experiments the muscle appears able to respond again sooner after a subnormal than after a normal response.

Apparently, then, what determines when the muscle shall give a second or third response is not the transmission time of the disturbance coming from the nerve, as conditioned by the modified state of the conducting path, but rather the refractory period of the muscle itself. We are thus led to the view that the nerve impulse in some way maintains a source of stimulation for a time after its arrival at the junction, and the muscle responds as soon as it is able, provided the source of stimulation has not died away. Clearly the muscle responses cannot follow each other with as brief intervals as can the nerve impulses. For instance, at 20°C. the nerve impulses can follow each other with intervals of 5σ or less, whereas the muscle responses seem unable to occur with briefer intervals than 6.5σ. If a prolonged series of nerve impulses occurs with a frequency that the muscle responses cannot follow, obviously the identity of the successive muscle responses with the nerve impulses of the same serial number will soon be lost. The question arises, how long after the arrival of a nerve impulse can it evoke a muscle response? When does the progressively increasing lag of the muscle responses behind the nerve impulses reach its limit, so that the muscle fails altogether to respond?

To answer this question we went a step further, and in eight experiments applied four stimuli to the nerve. It was difficult to establish the necessary experimental conditions of maximal first stimulus, maximal summation with second, and maximal additional summation with third, without fatiguing the preparation to a marked extent. The inequality of refractory period in individual muscle fibers complicates this case even more than that of three stimuli, and must be dealt with by the same sort of reasoning (see p. 594). No control by the size of mechanical contraction can insure the presence of four separate responses in any individual fibers, as was possible with three stimuli; for even if the contraction in response to all four stimuli is greater than that evoked by either stimuli 1, 2 and 3 or 1, 2 and 4, this might be due to the combined effects of some fibers responding only to stimuli 1, 2 and 3, some responding only to stimuli 1, 2 and 4, and others responding only to 1 and 3, depending on the length of the refractory period. In three separate experiments at 18.5°, 20.5° and 21.0°C., even though the contraction evoked by all four stimuli was in each

case greater than that evoked by any combination of three, the galvanometer records seemed to show that no muscle fibers had responded to all four stimuli. The evidence of this was that the fourth electric response followed the fourth stimulus with little or no greater latency than the first, although the latencies of the second and third responses were progressively greater. Thus at 18.5°C., with four stimuli separated by the following successive intervals, 7σ, 7σ and 9.3σ, four responses appeared in the record with intervals of 10σ, 9.3σ and 4.9σ respectively; the last response-interval was actually less than the corresponding stimulus-interval. Clearly, if our inferences from the experiments with three stimuli as to refractory phase are justified, the response to the fourth stimulus must have been limited to fibers which failed to respond to the third. In none of these three experiments at temperatures near 20°C. (all performed in February) did we evoke four successive muscle responses *in the same fibers*, even though the intervals between the nerve impulses were such that each of the first three was able to evoke a muscle response, and even though the fourth nerve impulse was sent in at a longer interval after the third than the intervals preceding it.

In two other experiments at temperatures of 19.5°C. and 23°C., performed in April and June respectively, we succeeded in evoking four responses *in the same muscle fibers* with stimuli at intervals similar to those in the three experiments just cited. The evidence of this lay in the appearance of a well-defined fourth response following the third at an interval compatible with the refractory period of the muscle as judged by the preceding response-intervals, and consequently with a latency even larger than that of the third response. The results of two separate sets of observations in each of these experiments are shown in table 6. The response-intervals in the experiment of April 3 are the averages of measurements made on several successive tests with the same stimulus-intervals. In most of the records made in this experiment two responses to the fourth stimulus could be distinguished, one occurring without abnormal latency, the other with a latency greater than that of the third response. Evidently the first of these was the response of fibers unaffected by stimulus 3, and the second was a genuine fourth response; only the intervals pertaining to the latter are given in table 6. Figure 17 shows a record of the quadruple response in which the double response to stimulus 4 did not appear, together with control records preceding it. Probably the stimulus-intervals were great enough to enable nearly all fibers to respond to each of the first three stimuli.

The conclusion indicated by our experiments is that in some preparations at certain temperatures it is possible to evoke as many as

four responses in the muscle with nerve impulses arriving at such high frequency that each successive muscle response lags farther behind the nerve impulse which evokes it. The fact that this result was obtained with all three stimulus-intervals as brief as 5.0σ at a temperature of 19.5°C. in April, but was not obtained in a test with intervals of 5.5σ, 5.5σ and 8.4σ at 20.5°C. in February, suggests a seasonal difference in the frogs; but of course it would require confirmatory experiments to establish this point.

TABLE 6

DATE	TEMPERATURE	INTERVALS IN σ						EXCESS LATENCY OVER 1		
		1-2		2-3		3-4				
		Stimulus	Response	Stimulus	Response	Stimulus	Response	2	3	4
	°C.									
Apr. 3, 1923	19.5	5.0	7.0	5.0	6.7	5.0	6.5	2.0	3.7	5.2
		5.5	7.0	5.5	6.9	5.5	5.9	1.5	2.9	3.2
June 14, 1922	23.0	2.65	4.5	2.65	3.6	2.6	3.8	1.8	2.8	4.0
		2.95	5.3	2.9	3.6	2.8	3.3	2.3	3.0	3.5

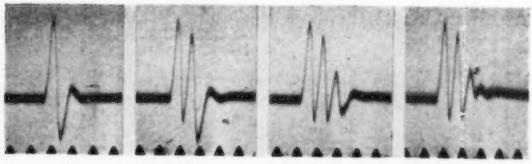


Fig. 17. Galvanometer records showing electric responses of muscle to four stimuli with progressively increasing latency.

April 3, 1923. Frog nerve-muscle preparation. Temp., 19.5°C. Stimuli applied to nerve; all successive stimulus-intervals, 5.5σ. Stim. 1, 10.5Z; stim. 2, 10Z (stim. 3 and 4, strength not determined).

A. Stim. 1 alone; B, stim. 1 and 2; C, stim. 1, 2, and 3; D, stim. 1, 2, 3, and 4. 17,000-ohm string, diam., 2.75μ; tension, 70 m. per amp. Magnification 350.

Discussion. We have sought to show that, considering the gradual restoration of the threshold during the recovery process, the simplest explanation of the time when a second response is made to appear in nerve or muscle by an early second stimulus, lies in the refractory phase of the tissue. We have suggested that the earliest time when a nerve can respond to a second stimulus depends on the duration of its refractory phase, and that the same is true of muscle, whether excited directly or through its motor nerve. We have sought to dis-

pense with the use of the term refractory phase as meaning the time of application of a second stimulus when it will be ineffective, since by suitable choice of experimental procedure this time can be made to disappear altogether. Instead we have used the term to mean the time when the tissue cannot respond. Our results have suggested that if a nerve impulse reaches the muscle during the refractory phase of the latter (using the term in our sense), it may yet evoke a response when the muscle has recovered sufficiently. To explain this we have been obliged to assume that the ability of a nerve impulse to excite the muscle persists for a time after its arrival at the junction. Referring to our experiments with three and four stimuli, it would seem that this ability to excite must endure for about 5σ at 20°C., and for about 4σ at 23°C. (see table 6).

On the mode of excitation of muscle by nerve Lapicque and Lucas have advanced conflicting views. Lapicque (34) contends that the nerve impulse constitutes the actual stimulus which excites the muscle. Lucas maintained that the disturbance is conducted from nerve to muscle through a distinct excitable substance at the junction. (31). According to Lapicque's view it would be necessary for us to show that that property of the nerve impulse which enables it to excite the muscle, lasts as long as the difference between the longest and the shortest latency we have found (e.g., 5.0σ at 20°C.). Following the well-founded suggestion that the action current is the essential mechanism of conduction, and construing Lapicque's views in this light, we may assume that the action current of the nerve is the stimulus which excites the muscle. To account for the maximum observed delay in transmission from nerve to muscle, the action current of the nerve should have a duration of at least 5σ at 20°C. Gasser and Erlanger (35) have shown with the Braun tube that the action current in the sciatic nerve of a bullfrog at 25°C. lasts as long as 5σ. At 20°C. the duration would be considerably longer, and the action current should persist with considerable intensity for 5σ. Adrian's electrometer records at lower temperatures suggest a shorter duration (9). We are unable to account for the apparent discrepancy, which may be due to a real difference between the nerves examined by the different investigators, or to the difference in the method of recording. In choosing between the two methods we should perhaps place more reliance on the Braun tube which records without lag, whereas the lag of the capillary electrometer is large and requires a process of analysis. This introduces a possible source of error, although Adrian's calibration curves indicate that the error is at most a small one (9).

Whatever the duration of the action current, there is the possibility that the activity of the junctional tissue, which Lucas has postulated,

provides the means whereby the nerve impulse can excite the muscle a considerable time after its arrival. It is conceivable, supposing a separate junctional tissue to exist, that its response is of longer duration than the nerve impulse, and that as long as this response endures it can excite the muscle. It is also conceivable that a single response of this tissue lasts only about a third as long as the nerve impulse, as is suggested by the brevity of its chronaxie, deduced from Lucas' observations (11), (cf. 30, p. 369), and that a single nerve impulse sets up in it a series of three or four successive responses. The final one might outlast the nerve impulse just long enough to provide the stimulus for the muscle at the observed time after the nerve impulse arrives. Thus the maximum observed delay might be explained even if it should prove that the nerve impulse in itself has not a long enough duration to account for it. We know of no direct way to test these possibilities.

Lucas offered several reasons for assuming the existence of a distinct excitable substance at the neuro-muscular junction (31, p. 66). Among these are the delay in the transmission of even a single propagated disturbance from nerve to muscle, the block established by fatigue, the action of curare and nicotine, and the phenomenon of Wedensky "inhibition." Perhaps more striking than all these is the evidence which Lucas himself obtained on exciting with currents of brief duration (36). The curve which he obtained correlating the strength of the minimal exciting current with its duration when the muscle was excited in the region of the nerve endings, showed a discontinuity which led to the conclusion that besides the muscle fibers and ends of the nerve fibers there was a separate excitable substance whose excitation time (chronaxie) was briefer than that of either nerve or muscle fiber. The recent work of Jinnaka and Azuma (37) with the pore electrode has shown that time relations, similar to those which Lucas ascribed to the junctional tissue, can be found in a part of the muscle where there are no nerve endings. The time relations appear to depend in part on the way in which the current is led into the tissue (i.e., current density at the electrode). This result appears to destroy the strongest evidence for the existence of a special junctional tissue. The question then arises whether the other evidence adduced to show the presence of such a tissue cannot also be explained in some other way, and thus enable us to simplify our conception of the junction by dispensing with the postulate of a special substance.

Wedensky inhibition was shown by Lucas (13) to depend on the subnormal magnitude of nerve impulses arriving at the junction with such frequency that each fell in the relative refractory period following its predecessor. He was able to duplicate the phenomenon by introducing a region of decrement in the nerve by means of alcohol and

concluded that fatigue caused the junctional tissue to conduct with a decrement and thus extinguish the subnormal impulses.

It seems to us that the Wedensky inhibition could as well be explained without the aid of a special junctional tissue, if the fatiguing effect of repeated stimulation could raise the threshold in muscle fibers without preventing them from responding altogether. Assuming with Lapicque that the nerve impulse is the actual stimulus for muscle, then with a certain rise of threshold in the muscle fibers a full-sized nerve impulse would still be able to excite, whereas the subnormal impulses arriving at high frequency would fail. This seems a simpler explanation than that which postulates a special tissue conducting with a decrement; and the fact that an artificial decrement can simulate the effect has no great force, since the essential condition—subnormal magnitude of the nerve impulses—would produce the effect just as readily with a high threshold in the muscle as with a partial block at the junction.

Another phenomenon which Adrian and Lucas examined and explained (6), basing their explanation in part on conduction with a decrement in the junctional tissue, was the summation of propagated disturbances. This occurred when fatigue had rendered a nerve-muscle preparation incapable of responding to a single stimulus applied to the nerve. If in this condition a second stimulus is applied during the supernormal phase of recovery of the nerve it will excite the muscle. They duplicated this effect, as they did the Wedensky effect, by establishing with alcohol an artificial block in the nerve, but in this case they made the block just great enough to prevent a single, full-sized impulse from passing. A second impulse, arriving in the supernormal phase, was then able to pass through the block. From this they inferred that in the fatigued preparations summation was due to the combined effect of conduction with a decrement in the junction and the supernormal state of the impulses.

In this case also it seems to us that a high threshold of excitation in the muscle fibers, combined with supernormal nerve impulses, would serve quite as well to explain the effect as conduction with a decrement. If fatigue raised the threshold to the point at which a normal nerve impulse was no longer an adequate stimulus, a supernormal impulse might still excite the muscle. In short, if fatigue can raise the threshold in a muscle, Lapicque's view of the neuro-muscular junction will fit the facts of both Wedensky inhibition and summation of propagated disturbances just as well as the hypothesis of a junctional tissue conducting with a decrement. Although recent observations by Cobb and Forbes (38) on human muscular fatigue seem to suggest that there is a junctional tissue, they may also be explained by fatigue raising the threshold of muscle more than it reduces the size of the electric response of which it is capable.

In this connection it may be significant that our observations on the interval that may elapse between the arrival of the nerve impulse and the latest time at which it can excite the muscle, are perfectly compatible with the view that the action current of the nerve is the stimulus which excites the muscle, provided we accept as correct the duration of the action current indicated by the experiments of Gasser and Erlanger.

We have yet to consider the cause of the correlation between stimulus-interval and response-interval which Lucas found in his experiments in which he stimulated the nerve and recorded the responses in the muscle. This he illustrated in figure 10 of his paper (2) with a curve of the same type as those in figures 1C and 6. To offer a possible explanation of such a correlation on the same principle that we have used in explaining the responses of the nerve itself (p. 575), it would be only necessary to show that the stimulus whereby the nerve excites the muscle rises to a maximum, not instantly, but in a measurable time, and then declines gradually through a long enough period. This is just the sort of course that the action current is shown to follow, both in the records of Gasser and Erlanger (35) and in those of Adrian (9). We could apply to the nerve-muscle preparation the same principle that we have illustrated in figure 9 as explaining the response of nerve; only the curves indicating the course of the local excitatory process produced by stimuli applied at various times would instead represent the course of nerve impulses arriving at various times. There would be another difference,—because the earlier nerve impulses arriving in the relative refractory phase would be subnormal, we should reduce the size of the action-current curves representing these impulses. The result would be to accentuate the bending back of the lower part of the correlation curve, and this agrees with the marked bending back which Lucas found. We have no quantitative data on the threshold of muscle and its rate of decline in the junctional region which would enable us to assert that the recovering threshold would fall below the existing electrical disturbance in the descending phase of the nerve's action current in a manner which would account for the correlation curve experimentally found. We can merely say that the general form of each of the known curves expressing the essential quantities is such as to render this hypothesis reasonable.

If all the facts known about the neuro-muscular junction could be explained on the assumption that the action current in the nerve is the stimulus which excites the muscle, the elimination of the junctional tissue as a separate excitable unit would be a desirable step toward simplicity.

The chief difficulty in the way of dispensing with the idea of the junctional tissue seems to lie in the experiments with various drugs,

such as curare and nicotine. As Lucas pointed out (31, p. 68), these experiments prove that in the junctional region there is something different from either nerve or muscle *per se*. Histologically we know that there is structure at the junction differing obviously from muscle and nerve fibers elsewhere. Conceivably, however, there is nothing there physiologically distinct. Indeed the junction may be merely a point of connection between the two at which either the nerve fiber or the muscle fiber or both are modified in form, and yet the action current in the nerve may be, after all, the actual stimulus for the muscle. For instance, we might regard one or both structures as modified in such a way as to permit the more efficient excitation of the muscle fibers by the approaching electrical disturbance in their nerve fibers. Lucas' "β curve," obtained with currents of brief duration sent through the entire muscle in the junctional region, might be explained if the muscle fiber were so modified at this point that the current density resembled that which Jinnaka and Azuma obtained with the pore electrode. There might also be greater permeability to certain drugs at the junctional part of the muscle fiber than elsewhere. This argument is too speculative to elaborate further, but it offers the suggestion that the evidence obtained with drugs may be due to local modification of nerve and muscle fibers at their junction rather than to a third, physiologically distinct, excitable tissue.

The best we can do is to leave the question open; and while we cannot claim that direct stimulation of muscle by nerve is proved, we conclude that the existence of a separate junctional tissue cannot be considered proved without further evidence.

Before leaving the subject of transmission from nerve to muscle we should note the apparent discrepancy between the observations of Lucas (2) and those of Samojloff (39) on the amount of delay found in the appearance of the response in muscle to an early second stimulus applied to its nerve. Lucas worked with a capillary electrometer; Samojloff with a string galvanometer. Samojloff, taking as the beginning of the second response the point at which the curve, instead of continuing downward from the peak of the first phase to the peak of the second, bent sharply upwards, found a delay corresponding closely with that reported by Lucas. On the other hand, when Samojloff took as the beginning of the second response the first divergence of his record obtained with two stimuli from that obtained with one, he found very little additional delay in the second response. A close study of Lucas' corrected capillary electrometer records suggests that his method was probably as sensitive in detecting small changes as that of Samojloff. A possible explanation of the discrepancy may depend on the point of application of the electrodes. Lucas applied

the leads to the middle and the tendinous end of the muscle (gastrocnemius). In the majority of our experiments we have done the same, and in some we have placed both leads near together at the tendinous end in order to reduce the size of response and prevent the excursion of the string being too large to be recorded on our narrow film. With both arrangements the delay in the appearance of the second response corresponded fairly closely with that obtained by Lucas. We have not found the slight early divergence near the peak of the first phase of the first response that appeared in Samojloff's records.

We have already shown that there is considerable delay between the time of arrival of the second nerve impulse at the junction and the time of appearance of the second muscle response. It is conceivable that if the nerve endings are included in the circuit a sensitive galvanometer may record their response as well as the response of the muscle. Samojloff states that his leads were applied to the middle and the tendinous end of the muscle. Possibly, however, he applied the proximal leads slightly nearer the proximal end than Lucas did. With his sensitive galvanometer and high magnification the action current of the distal portion of the nerve fibers may have caused the slight divergence of the combined curves from the curve of the first response alone. We have endeavored twice to test this point experimentally. We have led off from both the distal and the proximal ends of the muscle. We have, however, been unable to interpret our records with electrodes at the proximal end since even the response to a single stimulus takes on a complex character, probably because the propagated disturbance travels simultaneously in both directions from a point between the electrodes. Assuming our conjecture to be correct, it will probably take a very careful adjustment of the position of the leads to bring out the nerve response distinct from the response in the muscle fibers themselves. We have not succeeded in doing this, and must leave the point unsettled.

In the views we have advanced to explain the delayed appearance of the response of muscle to the early second stimulus applied to its motor nerve we seem to have invoked a variety of causes. We ascribed the delayed initial appearance of the second nerve impulse to persistence of the second local excitatory process till the end of the refractory phase. We showed that the second nerve impulse, if set up during the relative refractory phase, is conducted more slowly than normal and therefore lags progressively farther behind the first. We have suggested that the delayed appearance of the second muscle response was because the ability of the nerve impulse to stimulate the muscle fibers persisted long enough after its arrival at the junction to enable the muscle to respond on recovery from its refractory phase.

Diverse as these explanations may seem, they all involve the same principle; each depends on the refractory phase of the tissue in question; all assume that at every point the tissue responds to the existing stimulus as soon as it is able.

According to the membrane theory of conduction it is the action current of the active part of the fiber which constitutes the stimulus for the inactive part ahead of the disturbance. Extending this view to the transmission of the disturbance from nerve to muscle we look on the action current in nerve as the stimulus to muscle. At each point in the chain we look on the time of the second response as the moment when the recovering threshold at that point first coincides with the stimulus existing there.

On this view at the point of stimulation the local excitatory process initiates a propagated disturbance as soon as the recovering threshold of the nerve is low enough to render this possible. The slowed conduction along the nerve depends ultimately on the fact that during the relative refractory phase the nerve's response at each point must wait until the recovering threshold becomes low enough for the subnormal electrical disturbance in the region just before it to excite the nerve at that point. In transmission from nerve to muscle the refractory phase is longer than in nerve, and therefore an additional delay occurs, although electrically the mechanism is essentially the same here as elsewhere. This sums up the mechanism of transmission and the causes of delay in what seems to us the simplest interpretation of the facts. We have by no means proved that our assumptions are correct, but they seem to us reasonable as the basis of a working hypothesis.

Application to the reflex arc. Our observations bear on work that has been done on conduction in the reflex arc and on the discharge of nerve impulses from the central nervous system. Adrian and Olmsted (32) experimented with the flexion reflex in the decerebrate cat, observing the response of the muscle to two stimuli applied to the afferent nerve. They recorded the mechanical response of the muscle and also the electric response with a capillary electrometer, and found that whether the stimuli were applied to the afferent or to the motor nerve, the least interval between two stimuli at which they obtained summated contraction was about 1.8σ. When they examined the electric response of the muscle, they found that two stimuli at this interval evoked, in the case of the reflex, two responses with an interval of 6σ, whereas when the stimuli were applied to the motor nerve the response-interval was only about 3σ. The difference in response-intervals they ascribed to the central part of the arc, and inferred that the additional delay occurred there. They concluded that "if two impulses come through the arc they are spaced out to 0.006 second

apart, however short the interval between the two stimuli which set them up," and that impulses cannot traverse the center at a briefer interval than this.

This is not a necessary logical conclusion from the facts just described. In arriving at this conclusion the authors mention the time when "the second impulse appears in the muscle." This indicates a point of view which we have contended is misleading in that it implies an identity between the nerve impulse and the muscle response which it appears to have evoked, instead of looking on the muscle fiber as a separate unit which responds when it can to an existent source of excitation. It is not proved that in the experiment of Adrian and Olmsted the second nerve impulse traversing the motor nerve evoked the second response observed in the muscle, for the following reasons. We are not justified in assuming that the volleys of impulses set up in the afferent nerve by the individual stimuli maintain their identity in traversing the reflex arc. The experiments of Lutz (40) furnished evidence suggesting that impulses from many individual afferent fibers by virtue of their extensive branching in the center may, on reaching a single motor neurone, overlap in time in a way that would simulate a sustained source of stimulation to this neurone (see 30, p. 377). There is evidence (22) that with the briefest flexion reflex evoked by a single shock, the impulses in the motor neurones of the peroneal nerve reveal a dispersion in time; i.e., are measurably lacking in synchronism. Furthermore, the experiments of Sherrington (41) and one of Adrian and Forbes (16, p. 324) gave evidence that even a single volley of afferent impulses may evoke repetitive discharge of impulses in the individual motor neurones. There is good reason, then, to suppose that in response to a single afferent stimulus, motor neurones may be caused to conduct not one, but two or three impulses in rapid succession from the center to the muscle.

If this is so, it must affect the inference to be drawn from the experiments of Adrian and Olmsted. Adrian and Forbes found that an early second nerve impulse may be set up in a mammalian motor nerve, and yet fail to excite the muscle (16, p. 326). Quite conceivably, therefore, in the experiment of Adrian and Olmsted with two afferent stimuli one or more nerve impulses traversed the motor nerve between that which evoked the first and that which evoked the second response in the muscle. The additional delay, in that case, should not be ascribed to the center, but to the time required for full recovery of the muscle. This inference may appear at variance with their other observation that the muscle can respond again as early as 3σ after the first response, if stimulated through its motor nerve. But the ability of the muscle to do so may depend on the impulse in this

case falling late enough in the relative refractory phase of the nerve, and therefore being nearly enough full-sized to excite the muscle comparatively early in its relative refractory phase; whereas the motor impulses arising from reflex excitation may be so frequent as to be very small and therefore below the muscle's threshold until 6σ after the first response. We may analyze this hypothetical course of events as follows: The first afferent impulse evokes two impulses in a given motor neurone. The second follows the first very early in the relative refractory phase. Our experiments indicate that at body temperature the earliest time that a second nerve impulse can occur after the first is about 1.7σ (see fig. 7), and therefore we may assume this interval between the first and second nerve impulses. The second afferent impulse arrives at the center about 2σ after the first, but finds the motor neurone refractory as a result of its early second response. It therefore fails to set up a new motor impulse till 1.7σ after the second. Thus the third motor impulse (set up by the second afferent impulse) reaches the muscle 3.4σ after the first. The muscle is in its relative refractory phase, and unresponsive to subnormal impulses; the nerve impulse is subnormal and therefore excites only after a delay of 2.6σ. Thus we could explain the appearance of the second muscle response 6σ after the first. The additional delay of 2.6σ is little or no greater than would appear possible, judging from the delay we have found in the response of muscle to the fourth nerve impulse of a rapid series (table 6), even allowing for the difference of temperature.

We see no way to prove this the correct interpretation of the delay observed by Adrian and Olmsted, and indeed there are certain reasons for supposing it is not. First, the delay in the second response of muscle to double reflex stimulation may exceed that required for full recovery, since the temperature coefficient of the refractory phase is large. Second, this explanation presupposes that the first volley of afferent impulses evokes a repetitive discharge of motor impulses. Although several of Sherrington's experiments strongly point to this phenomenon, it appeared to occur in only one in the series of Adrian and Forbes, the flexion reflex in the remainder seeming to be characterized by single impulses in the motor neurones. Some experiments already undertaken on this point seem to indicate a refractory phase of 6σ in the center, but others are not wholly concordant. We must, therefore, leave this question unsettled, merely noting that beside Adrian and Olmsted's interpretation, there is an alternative interpretation based on the relative refractory phase of muscle, which is conceivable if not probable.

In a previous paper from these laboratories (42), the idea just developed,—viz., that of a rapid series of subnormal nerve impulses

some may fall in the refractory phase of the muscle and fail to excite it—was made the basis of an analysis proposed for voluntary contraction. In that paper it was reported that the frequency of muscular action currents in a voluntary contraction varied with the temperature of the muscle. This might have been due to some effect on afferent impulses arising in the muscle, but it appeared simpler to explain it as due to the dependence of the muscle's refractory phase on temperature. This explanation involved the supposition just discussed,—that the motor-nerve impulses occur at a higher frequency than the muscle responses. To reconcile this conclusion with the observations of Piper (43) that the muscle can respond to nerve stimulation with a higher frequency than is found in voluntary contraction, we invoked the gradual recovery of the muscle threshold during the relative refractory phase and the subnormal magnitude of nerve impulses arriving at high frequency, just as we have in the alternative explanation proposed for the observations of Adrian and Olmsted.

There are certain points in the paper on voluntary contraction (42) which require modification in view of Lucas' observations on delay and the considerations that have been discussed in the present paper. On page 244 it was stated that the nerve-impulse frequency must be as great as the least common multiple of the muscle frequencies found at different temperatures. This statement was based on the assumption that the muscle response must always begin immediately on the arrival of the nerve impulse. This is clearly not so. The evidence in table 6 seems to show that at 20°C. a nerve impulse can excite the muscle at any time from its arrival at the junction till 5σ thereafter. A rapid series of nerve impulses, therefore, would maintain a continuous source of excitation to which the muscle might respond at such frequency as its refractory phase would permit. The frequency of nerve impulses required to do this at a given temperature would be determined by the duration of the ability of each impulse to excite the muscle. From the data presented we may infer that at mammalian body temperature a frequency of about 300 per second would be required to sustain the excitatory source continuously at the junction.

According to the reasoning used in the analysis under discussion, this would mark the minimum frequency of nerve impulses involved in voluntary contraction. This agrees with the lower limit set in that paper on the ground that if the nerve-impulse frequency were lower the muscle responses would be able to follow them with the same frequency, instead of at the much lower frequency which has been repeatedly observed.

Another point in the paper on voluntary contraction needing revision is the upper limit set for the frequency of nerve impulses that

might be supposed possible. It was stated that this might be as high as 5000 per second. This statement was based on inferences drawn from the figures given by Adrian and Lucas (6, fig. 18) for the absolute refractory period of nerve at 15°C. and from the temperature coefficient of the refractory phase, found by Bazett (23). In drawing this inference an important fact was overlooked,—that the figures given by Adrian and Lucas referred to the refractory phase in the sense of the interval between *stimuli* below which a second response would not occur, and that the corresponding interval between *responses* was, as Gotch showed, much greater. Our experiments on the interval between nerve impulses at body temperatures seem to show that this is never less than about 1.6σ or 1.7σ. The inference from this would be that the greatest possible nerve-impulse frequency would be about 600 per second. This would alter the absolute quantities indicated in the diagram (42, fig. 5), and the difference between the nerve-impulse frequency and that of the muscle responses would be less than was represented there; but the principle remains unaltered.

We have shown that if a nerve impulse is initiated during the relative refractory period following a previous impulse, it travels more slowly than if the nerve had not been recently excited; therefore, the second of two impulses at this interval lags behind the first. For instance, at 35°C. impulses may be set up with an interval of 1.7σ, but the second cannot travel with normal velocity unless separated from the first by about 2σ. Therefore, if impulses are initiated at 600 per second they will tend to space themselves out till their frequency is 500 per second. If 600 impulses per second arise in the central end, with what frequency will they arrive at the muscle end of a long motor neurone? Will the frequency of arrival be limited there in consequence of the lag?

It is easy to work out the answer to this question by plotting the velocity of the third and fourth impulses in the same way that the velocity of the impulse shown by curve 2B in figure 11 was plotted. In consequence of the lag of the second impulse the third is forced to travel at subnormal velocity for a longer time than the second before it falls back to an interval which enables it to travel at normal velocity; the fourth must travel farther than the third before its lagging can cease, and so on. The ultimate condition reached with a prolonged series of impulses in rapid succession in a finite length of nerve must be one in which the impulses follow each other with the same frequency with which they were initiated, all being of subnormal magnitude and all travelling at the same subnormal velocity. Our experiments show the lagging to be so gradual that even at 13 cm. from the point of stimulation an early second impulse had not yet dropped far enough behind the first to travel at normal velocity (see fig. 13). Therefore

a very great length of nerve would be required to space out a rapid series of even three or four impulses enough to enable the last of the series to attain its normal magnitude and velocity. We may, therefore, conclude that in any case of sustained activity nerve impulses will not be appreciably spaced out in consequence of lagging, but may follow each other as rapidly as they are initiated, and continue to be of subnormal magnitude throughout their course. Certain experiments of a different sort, soon to be published, tend strongly to indicate that such a condition exists in certain reflex activity of a sustained character.

We may conclude from various lines of evidence that the frequency of motor-nerve impulses involved in sustained central activity, such as that which mediates voluntary muscular contraction, probably lies between about 300 and about 600 per second.

SUMMARY

1. Lucas observed that when a muscle is stimulated directly or through its motor nerve, a second stimulus applied soon after the end of what he termed the refractory period evoked an electric response with a longer latency than that of the first response. Gotch had previously reported a similar observation in the electric response of nerve to two stimuli. Lucas examined the correlation of the time of second response with the time of second stimulus in his own observations and in those of Gotch, and concluded that the extra delay of the second response was in each case caused by a modification of the tissue resulting from the previous propagated disturbance. He objected theoretically to the explanation which Gotch proposed,—that conduction was retarded during the recovery from a previous response; but he left unexplained the mechanism of the additional delay.

2. With both amphibian and mammalian nerves and nerve-muscle preparations we have repeated some of the experiments of both Gotch and Lucas, and have supplemented them with others. With a string galvanometer we have explored the nerve-muscle preparation, recording the responses to two stimuli of various strengths and at various intervals. We have recorded the responses of the nerve at various distances from the point of stimulation at various temperatures. We have recorded also the responses of the muscle to stimulation through the nerve.

3. With nerves we find that if the second stimulus is made sufficiently strong the refractory phase, in the old sense meaning the time at which the second stimulus may be applied without evoking a response, can be reduced to nothing. No stimulus is absolutely instantaneous. A break shock from an ordinary coil with an iron core may have sufficient

duration and strength to evoke a second response in nerve without any second stimulus. There is, however, a definite time after a response during which the nerve cannot respond again. The second response does not appear till the end of this time, whether evoked by a second stimulus or by virtue of the strength and duration of the first. We propose to call this time the refractory phase.

4. We have confirmed Bazett's observation that the strength of the first stimulus does not affect the recovery curve of the threshold, provided it be maximal in every case (i.e., adequate for all fibers). From this we infer that when the local excitatory process has initiated a propagated disturbance it ceases to exist.

5. By using a strong second stimulus we have shown that the interval between responses in the nerve is correlated with the interval between stimuli in a manner which Lucas found only in the case of a muscle excited though its nerve (see figs. 1C and 6). This type of correlation is most easily explained by the fact that an early second stimulus applied when the nerve is refractory can set up a local excitatory process which may persist till the nerve is sufficiently recovered to respond again, and by the fact that the threshold recovers gradually during the relative refractory phase. This explanation is supported by the observation that at a given stimulus-interval a large increase in the strength of the second stimulus hastens the second response.

6. Using long nerves from the cat and the bullfrog, and applying two pairs of leads to the nerve, one near to, and one far from the point of stimulation, we have shown that a second impulse set up in the relative refractory period travels slightly more slowly than the first. This observation has been corroborated by more accurate measurements of Gasser and Erlanger with the Braun tube. The speed approaches normal gradually as the interval is increased, and becomes normal at about the same interval at which the size of the second response becomes normal. This slowing of conduction can be simply explained, on the membrane theory of the nerve impulse, in terms of known features of the relative refractory phase, and thus tends to support the membrane theory.

7. In recording the response of muscle to stimulation through its nerve we find that in addition to the delay in initiating the second nerve impulse and the slight lag due to its slower conduction there is a distinct delay in the setting up of the second muscle response by the second nerve impulse. This is most simply explained as the result of the refractory phase of the muscle, which prevents it from responding sooner. This demands the postulate that the nerve impulse has the power to excite the muscle for a time after its moment of arrival at the neuro-muscular junction, either by virtue of its own duration, or through some special activity to which it gives rise at the junction.

8. This last conclusion is reinforced by records showing a still greater delay in the response of the muscle to a third nerve impulse. The time when the third muscle response appears is most simply explained as depending on the muscle's refractory period. This latter appears to be briefer after a subnormal response than after one of normal magnitude. There seems to be no need of assuming any modification of a tissue other than the refractory phase resulting from the passage of a propagated disturbance.

9. With certain nerve-muscle preparations at certain temperatures it is possible to evoke four successive muscle responses to four nerve impulses in such rapid succession that each muscle response shows a greater delay than its predecessor. By this means it is shown that the muscle can start to respond to a nerve impulse at any time after its arrival during an interval approximately equal to the duration of the action current of the nerve. This tends to support the view that the action current of the nerve is the stimulus for the muscle. In this connection the possibility of explaining Wedensky "inhibition" and summation of propagated disturbances on Lapicque's theory of direct excitation of muscle by nerve (without the intervention of a special junctional tissue) is discussed. The question is left unanswered.

10. The bearing of these observations on certain reflex activities and on voluntary contraction is considered. It is shown that the refractory phase of muscle may possibly explain certain observations on double reflex responses which have been taken to signify a long refractory phase in part of the reflex arc. The bearing of this on the frequency of motor-nerve impulses involved in sustained voluntary contractions is discussed. It is concluded that this frequency probably lies normally between 300 and 600 per second.

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REFLEX HYPERGLYCEMIA: A STUDY OF THE CARBOHYDRATE MOBILIZATION EFFECTED BY AFFERENT CRURAL, SCIATIC AND VAGUS STIMULATION[1]

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Studies of the nervous control of carbohydrate mobilization have usually made use of Claude Bernard's piqûre, or of some form of stimulation of the splanchnics or their terminations within the abdomen. But the unphysiological character of a stab in the floor of the fourth ventricle hardly needs to be emphasized; and there is abundant evidence in the literature, and to be cited later, that such manipulations within the abdomen as must necessarily accompany direct splanchnic stimulation are liable, of themselves, to produce a situation that may seriously obscure the effect of the special stimulation that is being tested.

If the mechanism controlling carbohydrate mobilization is under nervous control, it would seem possible that its activity might be modified reflexly. In fact, such a form of control is the only one of any direct physiological significance and for this reason alone is worthy of serious consideration. And fortunately, as an experimental method it is superior to the means usually employed; for with proper precaution the sciatic, crural or vagus nerves may be exposed and prepared for stimulation without causing any serious disturbance of the blood sugar level in a properly anesthetized animal.

For this reason it is surprising that so little work has been done on the reflex control of carbohydrate mobilization. The effect of afferent vagus stimulation on the blood sugar level has been studied somewhat (20), but not sufficiently to completely clarify the reaction; and although it has long been known that stimulation of the sciatic nerve is capable of causing glycosuria (1, p. 318), yet its effect on the blood sugar level seems never to have received any special attention, nor in any other respects has the mechanism of the reaction been investigated.

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The purpose of this paper is to place on record some data concerning the mechanism of the reflex hyperglycemia produced by afferent sciatic, crural and vagus stimulation.

PRELIMINARY DATA

1. METHODS. *Animals.* Rabbits were used for some of the work, but cats were employed in most of it.

Nutrition of the animals. In no case were animals used immediately after being brought to the laboratory, but only after they had had all they wanted to eat for several days in the laboratory pens, and never unless they seemed to be in a good condition of nutrition. No special diet was used during this preliminary period, but the cats had all they wanted of milk and meat and the rabbits had an abundance of the usual regimen of hay, oats and carrots.

In order to obtain anything like constant blood sugar values it was found necessary to work only with animals that were not actively digesting at the time of the experiment; this necessitated an 8 to 12 hour fast for the cats and an 18 to 20 hour fast for the rabbits preceding the experiment.

Temperature. The experiments were performed in a closed room of practically constant temperature. In addition, an electrical heating pad under the animal made it possible to maintain a practically constant and normal rectal temperature. At the most, the animal's temperature rarely varied more than a degree or two Centigrade, throughout an experiment of several hours duration, and such variations were so gradual when they did occur that they could not possibly obscure the results.

Blood sugar. In none of this work was attention paid to the appearance of sugar in the urine, but variations in the amount of sugar in the blood were employed, exclusively, as the criterion of carbohydrate mobilization.

The collection of the blood sample: Rabbits. In most of the work on rabbits the blood sample was obtained from the marginal ear vein after clipping the hair as closely as possible, washing the ear with soap and water, rinsing with water, alcohol and ether.

For the collection of samples from an unanesthetized animal, it was placed in a box just large enough to hold the body without cramping and provided with an opening of adjustable size through which the head could be brought and held without discomfort. The marginal ear vein was pricked with a needle or sharp scalpel sufficiently to allow the blood to flow out in a rapid series of drops. These drops were caught in a small, flat-bottomed, glass vessel, 15 mm. in diameter and 5 mm. deep and containing a few small crystals of potassium oxalate.

Although the potassium oxalate was not measured accurately, only a very small quantity was used and the effort was made to use as nearly as possible identical amounts in the different samples; for this purpose finely pulverized oxalate was kept in a bottle, from which it was removed by a rod, flattened at the end so as to form a small spatula; when this rod was dipped in the pulverized oxalate a small amount of "dust" adhered to it, when it was carefully withdrawn, and could be dislodged into the dish by a slight tap.

As the blood collected in the dish it was stirred with a nickle-plated rod to ensure even mixture of the anti-coagulant. After enough blood had been collected (slightly over 0.5 cc.) the bleeding was easily stopped by applying a little cotton to the wound; this was left in place to form a clot while the blood was disposed of (see below); it was then removed and the wound and adjacent areas washed with water, alcohol and ether; a subsequent sample could be obtained by opening the old wound or making a prick in a new area.

The collection of blood samples from the marginal ear vein of anesthetized rabbits was done in this same way after tying the animal down. Under anesthesia, however, the blood often flowed too slowly unless the cervical sympathetic had been cut on the side that was being used; so this procedure was regularly employed after anesthetization.

In some cases the blood was collected after anesthetization from the femoral artery, as from cats, in the following manner.

Cats. Blood samples were collected from cats only after anesthetization and tying down. For this purpose a glass cannula was tied in the femoral artery just distal to its saphenous branch, after exposing this portion of the artery through the smallest possible incision and with the greatest care to avoid any unnecessary trauma and stimulation of the closely lying crural nerve. After its exposure an artery clamp was placed on the artery just distal to the point of bifurcation and as near it as possible; the cannula was then inserted just distal to the clamp; by this means no "dead space" was left proximal to the clamp in which blood could stagnate between the withdrawal of samples; whatever blood entered the cannula should, therefore, be a representative sample of the general arterial blood at the time. The cannula was only large enough to hold slightly more than 0.5 cc. of blood and was of sufficient diameter to admit the end of the pipette that was used to take up and measure the sample.

In obtaining a sample a small amount of potassium oxalate was placed in the cannula in the way that has previously been described, the artery clamp was released enough to permit the cannula to fill with blood (just a little more than 0.5 cc.); the blood was immediately stirred with a small nickle-plated rod to ensure even mixture of the anti-coag-

ulant. After the blood was removed and disposed of (see below) the cannula was first cleaned with a small amount of dry cotton held in long, slender forceps; then washed out with a wad of cotton, moistened with water and rolled to a thread at the end, so as to clean out the neck of the cannula; then dried with dry cotton, similarly rolled out at the end so as to go into the neck of the cannula.

The analysis of the blood sample. The method of analysis was that of Folin and Wu (2) with such modification as to quantities of reagents used, as the small sample necessitated.

After a blood sample was obtained in either of the ways just described, it was immediately taken up in a 1 cc. pipette, graduated in hundredths of a cubic centimeter, and exactly 0.5 cc. of the blood added to 3.5 cc. of distilled water, previously measured into a test tube; after time for laking, the proteins were precipitated by the addition of 0.5 cc. of the sodium tungstate and 0.5 cc. of the $\frac{2N}{3}H_2SO_4$ solutions. In order to obtain from this 5 cc., the 2 cc. of filtrate which are required for use with the special test tubes recommended by Folin and Wu, it is necessary to use 55 mm. filter paper (Whatman no. 1 was used in this work) and specially selected, longnecked, small-caliber funnels. If no larger paper is used, and if the funnel is good, there is no difficulty in obtaining slightly over 2 cc. of filtrate; as soon as filtration was completed, exactly 2 cc. of this filtrate were taken up in a 2 cc. pipette and transferred to the special 25 cc. test tubes in which the remainder of the process was carried out as described in the original method.

Eight or ten such samples (rarely as many as twelve) might be taken in the course of an experiment lasting from three to five hours; the 2 cc. samples of the protein-free filtrate were therefore kept in stoppered test tubes until the end of the experiment; reduction was then carried out in all of them at the same time and they were all compared with the same standard.

Every care was taken to ensure that the results should represent absolute values within the ordinary limits of error of the method, but the primary requirement for this work was to have the samples faithfully represent the relative blood sugar values at the different stages of the experiment. For that reason it has not been thought necessary to undertake a justification of the use of this method of analysis. Aside from the probability that it is as accurate as any, it is certainly quick and convenient and capable of giving accurate relative values. Since correct relative values were wanted, it seems worth while to emphasize, also, that all details of the work were as carefully standardized as possible; it has already been mentioned that, without being unnecessarily meticulous, care was taken to have about the same amount of oxalate

in the different blood samples; they were laked for the same length of time; the time between precipitation and filtration was the same; and, as has been mentioned, they were all reduced simultaneously and quantitated against the same standard; it has also been mentioned that the greatest care was taken to clean the cannula thoroughly after each sample and in preparation for the next; and finally an effort was made to collect the samples at about the same rate. These are all small matters, but for that reason they are more easily done methodically than otherwise.

This method of blood analysis did not permit duplicate determinations to be made on the same blood sample. This might have been a serious objection if absolute values had been important. But variations within the limits of error of the method were of no significance in this work and greater reliance was placed on a large number of determinations obtained from numerous experiments than on a few determinations carefully made in duplicate. And aside from that, the use of small samples is more than justified by the small, total amount of blood thus necessarily lost by the animal. In this way the blood sugar variations could be carefully followed during an experiment of several hours duration without necessitating a total hemorrhage of more than 5 to 6 cc. There is enough evidence in the literature to indicate strongly that hemorrhage may, of itself, disturb the blood sugar level; but whether one believes this evidence is conclusive or not, it is worth while avoiding a possible source of error if it can be done without introducing a more serious one.

"Normal" blood sugar and the criterion of hyperglycemia. The question as to what constitutes a hyperglycemia is one of the most serious problems in connection with blood sugar work and the lack of an adequate criterion has probably been responsible for no small amount of the confusion in the literature on this subject. This condition of affairs is no doubt due to the difficulty of establishing "normal" blood sugar values for the different experimental animals. A review of the data at hand even makes one doubt whether the "normal" blood sugar value (if there really is any such thing) has ever been determined for any animal (3, pp. 30–33), (4). But, further, the work to be described here, like most of the work on the experimental hyperglycemias, has involved a certain amount of handling of the unanesthetized animal with whatever emotional disturbance and fright may be thereby occasioned; in addition, the animals have then been anesthetized and very often the experiment has involved a considerable amount of operation. Whether one is completely convinced or not, there is certainly enough evidence in the literature to show that such factors *may*, themselves, cause serious hyperglycemias. At all events, it is beyond dispute that an animal in such a condition is not *normal;* so it is at least seriously doubtful

whether "normal" blood sugar values could have any application in such work, even were they known.

A commendable effort has been made (5), (6) to meet this situation by establishing carefully determined data from which the effect of any of these routine experimental factors can be roughly evaluated and allowed for. But the most conspicuous feature of the data accumulated for this purpose is their lack of uniformity; so that it becomes extremely difficult to know what allowance to make for the effect of the anesthetic and other factors and thus decide from averages or a comparison of two experiments as to the effect of the particular and special procedure which is being tested. In work of this kind nothing seems more definitely and clearly established than that the blood sugar level of an animal under experimental conditions is a highly individual and unpredictable quantity, even under as nearly uniform and standard conditions as it is possible to maintain and reproduce. Not only does the blood sugar value vary greatly from one animal to another, but its variations in any one animal must be known before one can say with assurance that any special procedure has caused the level to rise or fall (6, p. 416).

For these reasons the effort was always made in these experiments to establish the general trend of the blood sugar variations before the effect of any special procedure on the blood sugar value was tested. In order to do this, at least two blood samples were taken, as a rule at 15-minute intervals, just preceding the test. In this way it was possible to know definitely, in each individual instance, whether the special procedure being tested caused any variation in the blood sugar "curve."

But although it is believed that such a method largely reduces the chance of anesthetic and operative effects obscuring the results, it does not obviate the necessity of exercising care in regard to these factors; for if they are capable of producing a hyperglycemia, i.e., a mobilization of the carbohydrate reserves, they will, to that extent, reduce the apparent efficacy of any other procedure tending to have the same effect (see fig. 2). For this reason the choice and use of an anesthetic and information as to the disturbance caused by the operations employed, are matters of fundamental importance.

Anesthetic. Unless otherwise mentioned, chloralose (7) was used as an anesthetic in these experiments; after some preliminary experience with ether (see fig. 2) and urethane, this anesthetic appeared to be best for this work, because it seemed to produce less disturbance of the blood sugar level than the others. It is superior to ether on this account and also because it may be given intravenously, or *per os*, and thus the respiratory disturbances that are so difficult to separate from etherization may be avoided; given in either of these ways, it also establishes a a more uniform anesthesia than it is possible to maintain with ether; and

this is important in experiments of several hours duration, during which it is desirable to eliminate as many variables as possible. Compared with other anesthetics that may be similarly administered (e.g., urethane), chloralose seemed to produce less hyperglycemia. Whether it produces any hyperglycemia or not is, of course, very difficult to determine and no experiments directly aimed at this point have been undertaken.

In the work with rabbits, the injection of the anesthetic intravenously (10 cc. of 1 per cent chloralose in mammalian Ringer per kilo) in the marginal ear vein, always produced an immediate rise in the blood sugar level which reached a maximum about an hour after the injection and had fallen to a lower and uniform level at the end of two hours (see figs. 1A and 4A); this level was then maintained with so much uniformity for three or four hours subsequently, that it provided a good background against which to test the effect of any special procedure on the blood sugar value. In working with rabbits, therefore, it was customary to wait for an hour and a half or two hours after injecting the anesthetic before doing anything further to the animal.

No effort has been made to determine whether this initial hyperglycemia is due to the anesthetic itself; or to the amount of salt solution (Ringer) in which the anesthetic was dissolved and which was therefore injected at the same time; or to the excitement attendant upon pricking the ear with the needle and making the injection. That the effect may not be due to the anesthetic itself is, perhaps, suggested by the fact that the blood sugar subsequently falls, although the anesthesia is apparently not diminished; and further, the effect is quite different in cats, with a different mode of administration.

Unless otherwise stated, the drug was given to cats, *per os*, the required amount (0.1 gram per kilo) being shaken up with 25 cc. of milk. In only a few cases was it necessary to use a stomach tube and this was avoided whenever possible because of the concomitant struggling and excitement usually associated with its use. As a rule the cat, which had not eaten anything for 8 to 12 hours, drank the milk eagerly and licked up any of the anesthetic that had not dissolved and had settled out in the dish. This method has the highly desirable result of producing a condition of complete anesthesia without any excitement or disturbance whatever, beyond what may be produced by the strange environment itself; and the blood sugar level of cats, anesthetized in this way, was regularly lower than that of the rabbits which received the anesthetic intravenously (see figs. 1B and 4B).

The value of chloralose for this work seems, therefore, to be that it does not produce as much disturbance of the blood sugar level as other anesthetics are liable to do; and the level is rather remarkably constant throughout the period of anesthesia. Attention has been called to this last point by others (8) since this work was well under way.

2. The effect of operative procedures. The usual operations employed in this work were: exposure of the femoral artery and insertion of a cannula; insertion of a tracheal cannula; laparotomy followed by dissection and section of the nerves in the hepatic pedicle (denervation of the liver) or by removal of one adrenal and denervation of the other (by sectioning the homolateral splanchnic nerves and any connections with the lumbar ganglia).

With regard to the insertion of the artery cannula, no direct evidence is available, although some indirect evidence to be cited in a later paper would seem to show that this (perhaps together with the "fesselung") is sufficient to raise the blood sugar value considerably.

The effect of the insertion of a tracheal cannula was noted in only one instance (cat, exper. 58) and was then found to raise the blood sugar from 143 to 152 mgm. per 100 cc. It might be mentioned also that the

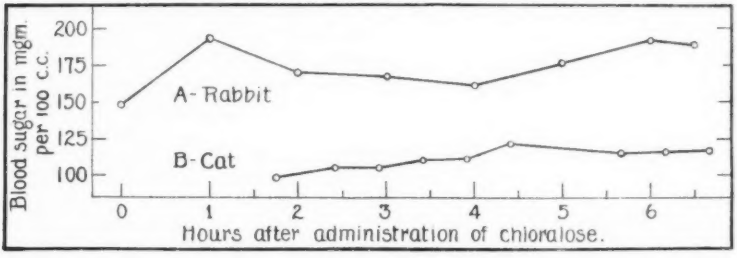

Fig. 1. The effect of chloralose on the blood sugar. A: The average of six experiments in which, at *0*, 1 per cent chloralose in mammalian Ringer, 10 cc. per kilo, was injected in the marginal ear vein of rabbits. B: A single experiment with a cat in which, at *0*, the cat drank 0.1 gram of chloralose per kilo, in 25 cc. of milk.

effect of a more extensive operation in this same region, viz., bilateral thyroparathyroidectomy and section of both cervical sympathetics, was noted in one case (cat, exper. 48) and found to raise the blood sugar from 136 to 151 mgm. per 100 cc.

Operations of this kind, however, seem to be in no degree as disturbing as those which involve opening of and manipulations within the abdomen. The serious effect of merely opening the abdomen has been called attention to before (9) and is reported to produce a greater hyperglycemia than the amputation of a leg. In the only experiment of this series in which the effect of merely opening the abdomen was tested, it was found to raise the blood sugar value from 169 to 224 mgm. per 100 cc. (cat, exper. 24).

Tables 1 and 2 (see also figs. 5 and 6A) show the effect on the blood sugar value of two types of abdominal operations that are of rather common use in work on the experimental hyperglycemias.

TABLE 1

The effect of adrenalectomy on the blood sugar level of chloralose-anesthetized cats

EXPERIMENT NUMBER	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR INCREASE IN PER CENT	REMARKS
	Before operation	After operation	Increase		
18	465	555	90	19	Laparotomy and removal of both adrenals
20	177	224	47	25	Laparotomy and removal of both adrenals
21	181	296	115	63	Laparotomy; right adrenal removed; left splanchnics cut
22	206	336	130	63	Same as preceding
24	133	181	48	36	Both adrenals removed by dorsal (retroperitoneal) route
25	240	363	123	51	Both adrenals removed by dorsal (retroperitoneal) route; the hepatic nerves had been cut 5 weeks previously
27	111	170	59	53	Laparotomy and removal of left adrenal; the right adrenal had been removed, the left splanchnic nerves cut, and the nerves in the hepatic pedicle cut 27 days previously
28	81	277	196	241	Same as preceding except that the experiment was 25 days after the previous operation
30	209	357	148	70	Laparotomy, removal of right adrenal, left splanchnics cut; the nerves in hepatic pedicle were cut 5 weeks previously
31	140	217	77	55	Same as preceding except that the experiment was 4 weeks after the previous operation

TABLE 2

The effect of dissecting and cutting the nerves in the hepatic pedicle of chloralose anesthetized cats

EXPERIMENT NUMBER	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR INCREASE IN PER CENT	REMARKS
	Before operation	After operation	Increase		
14	275	347	72	26	Both adrenals were intact
15	206	425	219	106	Both adrenals were intact (see fig. 5)
16	370	481	111	30	Both adrenals were intact
21	227	281	44	19	Right adrenal had been removed and left splanchnics cut 1 hr. 20 min. previously
22	347	362	15	4	Right adrenal had been removed and left splanchnics cut 2 hrs. 50 min. previously
32	122	263	141	115	Same as no. 22, but 6 weeks previously
33	173	229	56	32	Left adrenal removed and right splanchnics cut 5 weeks previously

In conclusion, attention must be called to what is probably the most important detail to be mentioned in this preliminary account. Not only may anesthetics and operations cause great hyperglycemias, but such disturbances of the blood sugar regulating mechanism, however caused,

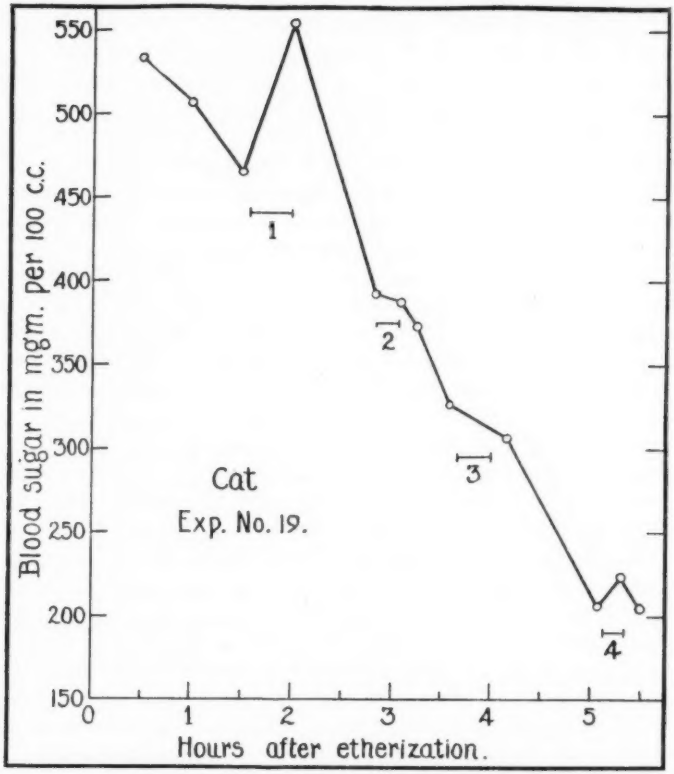

Fig. 2. The first blood sample was taken as quickly after the beginning of etherization as the insertion of a tracheal and artery (femoral) cannula allowed. At *1* the increase in blood sugar value was caused by laparotomy and removal of both adrenals. At *2* the central end of the left sciatic nerve was stimulated for 5 minutes. At *3* the nerves in the hepatic pedicle were dissected and cut. At *4* the central end of the left sciatic was stimulated as at *2*.

when once established, constitute an abnormal condition out of which it is extremely difficult or impossible to obtain normal results. Figure 2 illustrates a case in point. This was one of the few experiments that was done under ether anesthesia and it is given here because it illustrates what seemed to be typical of this form of anesthesia and of the effect of

operative procedures. After an intense initial hyperglycemia (0.533 per cent) the blood sugar level began to fall; laparotomy and removal of both adrenals interrupted this fall, but only temporarily. But it is a comparison of *2* and *4* in this figure that is especially interesting; at both points the central end of the left sciatic nerve was stimulated with maximal induction shocks (tetanizing current) for the same length of time. It will be shown later that this is an adequate means of producing a hyperglycemia, but the point to note here is that although this was capable of causing a slight positive increase in the blood sugar value at *4* after the blood sugar had returned to a lower, or what we may think of as a more normal value (0.206 per cent; the stimulation raised it to 0.224 per cent, or an increase of 8 per cent), yet the same stimulation at *2* might seem at first glance to have had no effect. This would certainly have been the conclusion if the blood sugar "curve" preceding and following the stimulation had not been known. With this information, however, it is seen that the effect of the stimulation at *2* was not negative but was merely masked to a certain extent by the general situation. The same effect is shown at *3*, when the nerves of the hepatic pedicle were dissected and cut; table 2 shows clearly enough that dissection of the nerves to the liver will ordinarily cause a marked hyperglycemia; and inspection of the total curve of figure 2 shows that the effect was significant in this case; but, again, unless the entire "curve" had been thus determined it would have appeared that this operation really diminished the blood sugar value in this particular case, whereas what it really did was to cause a significant decrease in the rate of decline of the blood sugar value which was going on "spontaneously" at the time.

This is only one case out of a number that could be cited in this connection. And it is believed that such evidence justifies the contention that: *a*, in studying the mechanism of carbohydrate mobilization every precaution should be taken to disturb the blood sugar level as little as possible by incidental factors; but especially, *b*, under experimental conditions, the fluctuations in blood sugar value must be followed with some regularity before it can be known with definiteness what effect any special procedure has had on the amount of sugar in the blood.

THE HYPERGLYCEMIA PRODUCED BY AFFERENT SCIATIC OR CRURAL STIMULATION

The general conditions of these experiments were as described in the first section of the paper. Only the method of stimulation remains to be mentioned; the nerve to be stimulated (sciatic or crural) was never exposed until the time for stimulation; it was then quickly laid bare, with as little and as careful dissection as possible and placed on specially prepared, shielded electrodes of such a type as to slip over the nerve

without cutting it; then just before the electrical stimulation was begun the nerve was crushed with a hemostat distal to the electrodes so as to prevent any possible peripheral effect. This process of preparation should take less than a couple of minutes and whatever effect the operation might have on the blood sugar level is thus added to or lost in the immediately following effect of the electrical stimulation.

The stimulation was done with a tetanizing current from the secondary coil of a Harvard inductorium; the primary current was about 4.5 volts and 0.3 ampere and was interrupted for 5 seconds every 5 seconds, i.e., the stimulation was on for 5 seconds and off for 5 seconds, alternately; the secondary coil was usually placed at the 6 cm. mark on the scale of the inductorium. Such intensity of stimulation produced marked pressor effects the few times it was so tested (see table on p. 655).

Figure 3 shows the result of a single experiment in which the central end of the sciatic nerve of a cat was stimulated continuously (in the interrupted manner just described) for 55 minutes. From this experiment it is seen that the blood sugar response is prompt; the hyperglycemia produced by 5 minutes of stimulation is nearly maximal. For this reason the stimulation was rarely kept up longer than 5 minutes in these experiments.

Tables 3 and 4 contain the results of such brief stimulation with a number of rabbits (III) and cats (IV); figure 4 illustrates two typical cases.

Perhaps the most noteworthy feature in regard to the data in tables 3 and 4 is the entire lack of any uniformity in the blood sugar values. As far as could be determined the animals were all in an adequate state of nutrition, as explained in the first section of this paper, so that there is no obvious correlation with variations in this regard. There is much evidence that the magnitude of the glycogen stores undergoes a seasonal variation, but even if this is true, the variability in these results would not seem to be capable of such an explanation, since the work on rabbits was all done at practically the same time of year and that on cats at the two opposites of summer and winter. Neither the nerve stimulated, nor the time of stimulation after anesthetization, nor the duration of stimulation seem to account for the variability in any way. Of course, there was no way of judging the *relative* intensity of the stimulation with respect to the physiological condition of the animal at the particular time; but since the stimulus was definitely supramaximal and was of the same strength throughout, this only tends to emphasize the rôle of unknown and individually variable factors in influencing the results.

It is also worth while noting that not only the changes in blood sugar level produced by this form of stimulation, but also the "undisturbed" blood sugar values have no uniformity; this is true of the values of the

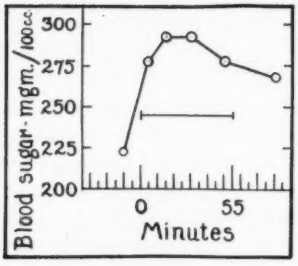

Fig. 3. The effect of long-continued (55 minutes) stimulation of the central end of the sciatic nerve of a chloralose-anesthetized cat (exper. 35). The blood sugar rose from 222 to a maximum of 292 mgm. per 100 cc.; at the end of the first 5 minutes of stimulation the blood sugar value was 279 mgm. per 100 cc., i.e., the main rise occurred during the first 5 minutes of stimulation.

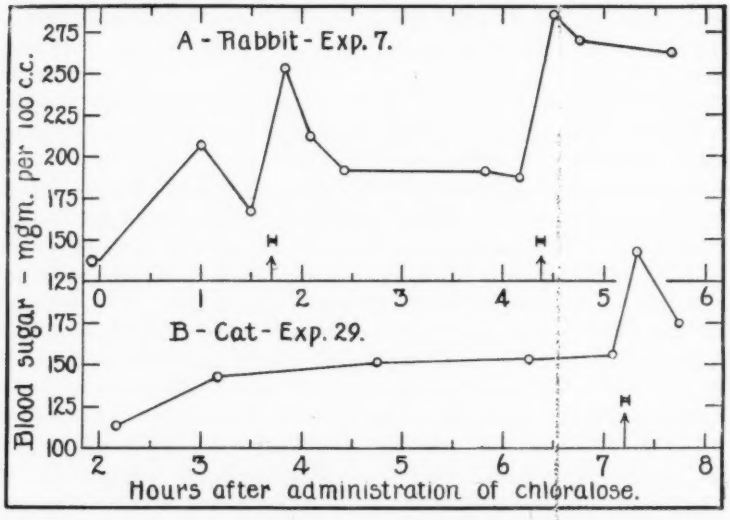

Fig. 4. The effect of brief, afferent stimulation on the blood sugar value. A: Rabbit; the central end of the right crural nerve was stimulated for 5 minutes (1:40–1:45; and 4:20–4:25). B: Cat; the central end of the left sciatic nerve was stimulated for 5 minutes (7:10–7:15). The actual changes in blood sugar value produced by these stimulations can be seen in tables 3 and 4.

Curve *A* shows the initial hyperglycemia produced by the injection of the anesthetic (see fig. 1A).

rabbits before and after anesthetization and of the cats immediately after anesthetization. Of course these animals can be spoken of as

TABLE 3

The effect of afferent crural and sciatic stimulation on the blood sugar level of normal, chloralose-anesthetized rabbits

EXPERIMENT NUMBER	DATE	BLOOD SUGAR IN MILLIGRAMS PER 100 CC. BEFORE ANESTHETIZATION	TIME OF STIMULATION AFTER THE ADMINISTRATION OF CHLORALOSE (HOURS)	NERVE STIMULATED	DURATION OF STIMULATION (MINUTES)	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR CHANGE IN PER CENT
						Immediately before the stimulation	Immediately after the stimulation	Change caused by the stimulation	
1	Nov. 4	142	2:00	Crural	5	155	194	+39	+25
			5:30	Crural	5	210	243	+33	+15
2	Nov. 8	162	2:00	Sciatic	5	243	277	+34	+13
3	Nov. 19	150	1:45	Crural	5	177	175	−2	−1
			4:20	Crural	5	190	198	+8	+4
4	Nov. 28	144	2:00	Sciatic	2	250	259	+9	+3
			7:00	Sciatic	2	229	322	+93	+40
5	Dec. 6	139	3:00	Sciatic	2	400	454	+54	+13
			6:00	Sciatic	2	200	263	+63	+31
6	Dec. 13		1:40	Crural	2	408	487	+79	+19
			5:00	Crural	4	256	370	+114	+44
7	Dec. 16	138	1:40	Crural	5	166	253	+87	+52
			4:20	Crural	5	188	285	+97	+51
8	Dec. 21		1:30	Crural	3	404	459	+55	+13
			4:30	Crural	5	421	449	+28	+6
9	Dec. 23	200	1:30	Crural	3	263	333	+70	+26
			4:00	Crural	2	287	357	+70	+24
10	Dec. 26		1:40	Crural	5	241	304	+63	+26
11	Jan. 9		1:45	Crural	2	388	425	+37	+8
12	Jan. 12		2:00	Crural	2	224	279	+55	+25
Average								+54	+22

"undisturbed" in only a very relative sense; but, at all events, whatever disturbance they were subjected to was far more uniform than would

be indicated by the dissimilar blood sugar values; the animals were carried from the animal house to the laboratory as gently and with as little frightening as possible; an initial ("normal") blood sample (column 3, table 3) might then be taken from the rabbits before the injection of the anesthetic. The cats were set free in the laboratory, allowed to drink their dose of chloralose in milk and left entirely to themselves until the anesthesia was complete; only then was the first blood sample taken (column 3, table 4). Yet, in spite of these efforts to treat the

TABLE 4

The effect of afferent crural and sciatic stimulation on the blood sugar level of normal, chloralose-anesthetized cats

EXPERIMENT NUMBER	DATE	BLOOD SUGAR VALUE, IN MILLIGRAMS PER 100 CC. OF THE FIRST BLOOD SAMPLE	TIME OF FIRST SAMPLE AFTER ADMINISTRATION OF CHLORALOSE (HOURS)	TIME OF STIMULATION AFTER ADMINISTRATION OF CHLORALOSE (HOURS)	NERVE STIMULATED	DURATION OF STIMULATION (MINUTES)	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR CHANGE IN PER CENT
							Immediately before the stimulation	Immediately after the stimulation	Change caused by the stimulation	
13	Jan. 10	197	1:00	1:50	Crural	5	177 (2*)	201	+24	+13
14	Jan. 17		1:30	1:45	Crural	2	261 (1)	275	+14	+5
20	Feb. 4	147	1:40	2:30	Sciatic	3	151 (2)	171	+20	+13
21	Feb. 24	145	3:20	4:25	Sciatic	4	114 (2)	218	+104	+91
22**	Feb. 28	205	2:00	2:35	Sciatic	2	212 (2)	206	−6	−2
23	July 5		1:45	2:00	Crural	5	243 (1)	377	+134	+55
24	July 8		1:45	1:50	Crural	5	88 (1)	133	+45	+51
26	July 10	87	2:15	7:00	Sciatic	10	161 (6)	166	+5	+3
29	June 6	113	2:10	7:10	Sciatic	5	156 (5)	217	+61	+38
35	June 23	168	3:15	4:30	Sciatic	5	222 (3)	279	+57	+25
37	Nov. 3	107	1:45	2:25	Sciatic	5	113 (3)	175	+62	+54
Average									+48	+31

* The number in parenthesis indicates that the blood sample immediately preceding the stimulation was the first, third, etc., taken after anesthetization.

** Cat unusually sluggish throughout the experiment; respiration slow and blood poorly aerated.

animals alike and with as little disturbance as possible, there is no uniformity in their blood sugar values.

These facts are of particular importance as regards the cats, because the further work, attempting to trace out the mechanism of this reaction, was done almost altogether on them. It is therefore important to recognize the highly variable character of the response in these perfectly normal animals. And the results seem to indicate clearly enough that each individual case needs to be considered on its own merits; although

averages have been computed for these reflex hyperglycemias and others will be computed for comparison with them, yet the data being averaged are altogether too divergent in value to make an average figure serviceable except for purposes of the most general comparison.

When this variability of response in normal animals is kept in mind and when it is recalled how many reasons there are for expecting negative results in this work—the animal may not have much carbohydrate in store; the anesthesia may be too deep; the stimulation may be relatively inadequate, etc.—a clean-cut, positive result is of special interest. In short, negative results, unless overwhelmingly predominant, have been considered of little importance in comparison with positive reactions, even though the latter may be relatively few in number. This is the point of view from which the results in the following sections of the paper have been evaluated and this attitude should be kept in mind in any criticism of the final conclusions.

FACTORS INVOLVED IN THE SCIATIC OR CRURAL REFLEX HYPERGLYCEMIA

1. THE RÔLE OF THE HEPATIC NERVES AND ADRENALS. *A. Reflex hyperglycemia after cutting the nerves to the liver.* In the experiments under this heading, the hepatic nerves were cut in the lesser omentum in such a way as not to disturb any other portion of the abdominal innervation. To perform this operation, everything in the lesser omentum except the bile duct, hepatic artery and portal vein was dissected free of these structures and cut; with some practice it is possible to clean up the walls of these vessels so that not a single visible nerve fiber remains. This operation, of course, does not interrupt the continuity of any nerve fibers incorporated in the walls of the artery and vein, and Macleod, especially, has insisted upon the importance of sectioning these (13, p. 66); only after ligating and cutting everything in the hepatic pedicle except the portal vein, and searing its walls, was he able to obtain constant (negative) results from splanchnic stimulation. But it scarcely needs to be insisted upon that such a procedure, which shuts off the arterial blood supply to the liver, hardly permits any conclusion as to the effect of merely *denervating* that organ; and more especially, the evidence at hand (10), (12) indicates that permanent occlusion of the hepatic artery is quickly fatal unless the liver has an adequate collateral circulation. It is obviously very difficult to know, therefore, just how long after such an operation one could expect normal results.

Cannon and Rapport, however, have found (11) that the sort of hepatic denervation which has been adopted in this work prevents splanchnic and reflex stimulation from producing the usual discharge of a cardio-accelerator substance from the liver; these studies, which

employed the denervated heart as a test object, probably represent the most delicate measurement of hepatic secretory activity that has been made; and since they show that the nervous control of this secretory function of the liver is abolished by this form of denervation, it seems reasonable to suppose that any possible nervous control of the glucosecretory activity of the liver would be interfered with in the same manner.

This point is so important, however, that it was specifically tested in an experiment which had best be cited in this connection, although it anticipates in some respects what will be more fully developed later.

Exper. 45; cat; ♂

August 16, 1922. Weight 3.4 kgm. Under ether anesthesia the following operation was performed aseptically: liver denervated, i.e., everything in the hepatic pedicle except the bile duct, hepatic artery and portal vein dissected free of these structures and cut; right adrenal removed; left adrenal denervated, i.e., the left splanchnics major and minor and twigs between the lumbar ganglia and adrenal cut.

September 27, 1922. Cat had recovered from the operation and was in good condition; weight 3.6 kgm.; the following experiment was then performed:

8:30—0.3 gram chloralose in 25 cc. milk drunk by the cat
10:30—Cat tied down and cannula put in femoral artery
10:50—Blood sample—93 mgm.
11:30—Blood sample—94 mgm. } sugar per 100 cc.
Left sciatic exposed and stimulated for 10 minutes
12:00—Blood sample—118 mgm.
12:10—Blood sample—118 mgm.
12:30—Blood sample—111 mgm.
2:15—Blood sample—110 mgm. } sugar per 100 cc.
Abdomen opened, electrodes placed in the scar tissue of the hepatic pedicle and stimulation with intense tetanizing current (4.5 volts, 0.3 amp., in primary of Harvard inductorium; coil distance 0 cm.) for 5 minutes.
2:30—Blood sample—114 mgm., sugar per 100 cc.
Autopsy: the liver appeared normal and it was impossible to see any nerve fibers by inspection of the scar tissue of the pedicle.

Table 5 shows that such electrical stimulation of the hepatic pedicle as was used in this experiment never fails to produce an intense hyperglycemia in case the nerves along the hepatic artery have not been previously interfered with; yet in this case there was practically no change at all—110 mgm. before and 114 mgm. after the stimulation—and this in spite of the fact that a previous reflex stimulation had raised the blood sugar value from 94 to 118 mgm. per 100 cc. (+25 per cent). Unfortunately a determination of the hepatic glycogen was not made in this case, but there are several reasons for supposing that the negative result, when the hepatic pedicle was stimulated, can not be attributed to a

deficiency of this substance; the animal's nutrition appeared to be excellent—it weighed 0.2 kgm. more at the time of the experiment than it did when operated on six weeks previously; and other experiments, to be reported later, have shown that the glycogen content of the liver may be within the usual limits after recovery from such an operation.

This experiment seems to show, therefore, that the method of hepatic denervation, as it was practised in these experiments, does destroy the central connections of the gluco-secretory fibers; and a further point worth noticing is that these fibers have apparently not regenerated within a period of six weeks.

At all events, it was not thought justifiable to interfere with the arterial blood supply to the liver, in experiments that it was hoped would throw

TABLE 5

The effect of electrical stimulation of the hepatic nerves (in the lesser omentum) of chloralose-anesthetized cats

EXPERIMENT NUMBER	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR INCREASE IN PER CENT	REMARKS
	Before stimulation	After stimulation	Increase		
23	198	333	135	68	Both adrenals removed 2 hrs. 45 min. previously
24	224	408	184	77	Both adrenals removed 2 hrs. previously
26	162	392	230	141	Both adrenals removed 30 min. previously
29	210	298	88	41	Adrenals intact
42	185	253	68	36	Both adrenals removed 50 min. previously; the cat was curarized and under artificial respiration

some light on the normal nervous control of carbohydrate mobilization; and it may frankly be said that if the results are not felt to be indicative of what might happen with a completely denervated liver (i.e., one having every nerve fiber cut, including those in the walls of the blood vessels) they at least throw light on the function of those numerous nerves which run alongside the hepatic artery in the lesser omentum. Personally, it is not thought that any such reservation is necessary and it will be considered that, possibly aside from minor vasomotor reactions, the liver is completely denervated by this operation.

The experiments are of two kinds: *a*, acute experiments in which the stimulation was tested shortly after the denervation (1 to 3 hours); and *b*, experiments on cats that had recovered from a denervation done aseptically a few weeks previously. The latter form of experiment was

resorted to because of the great hyperglycemia which may be caused by the denervation itself (table 2). The results from both types of experiment are brought together in table 6; and figure 5 illustrates the effect on the blood sugar level of dissecting and cutting the hepatic nerves, as well as the effect of a subsequent afferent stimulation.

Inspection of the table shows that, with the exception of experiments 31 and 36, the reflex hyperglycemias produced by afferent sciatic or crural stimulation, after denervation of the liver, are individually of the same order of magnitude as those produced in normal animals; and even counting these two negative results, the average hyperglycemia obtained in this series of experiments is practically the same as that obtained with

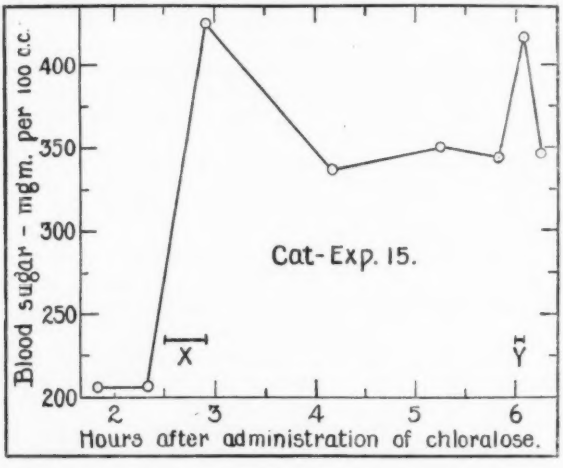

Fig. 5. The effect on the blood sugar level of a chloralose-anesthetized cat of dissecting and cutting the hepatic nerves, *X*, and of stimulating the sciatic nerve for 5 minutes, *Y*, after the liver has been denervated.

normal animals. This result may mean either of two things: it may be, as has been vigorously maintained (14), that glycogenolysis in the liver is not regulated by gluco-secretory nerves, but is effected by changes either in the quantity or composition of the blood flowing through the liver; or, the increase in the blood sugar value which takes place as a result of afferent crural or sciatic stimulation may not depend upon a mobilization of hepatic glycogen. At present it is impossible to decide between these alternatives, but the evidence points to the conclusion that denervation of the liver, as practised in this work, seems to have no effect on the increase in blood sugar value which may take place as a result of afferent sciatic or crural stimulation.

B. Reflex hyperglycemia after removal or denervation of the adrenals. The experiments of this series were of three kinds: acute experiments in which the stimulation was done within one to two hours after *a*, removing both adrenals; or *b*, removing one adrenal and denervating the other; and *c*, experiments on animals that had recovered from the aseptic re-

TABLE 6

The effect of afferent (sciatic and crural) stimulation on the blood sugar level of chloralose-anesthetized animals, after denervation of the liver

EXPERIMENT NUMBER	NERVE STIMULATED	DURATION OF STIMULATION (MINUTES)	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR CHANGE IN PER CENT	REMARKS
			Before stimulation	After stimulation	Change		
11*	Crural	3	454	540	+86	+18	Stimulation 1 hr. 50 min. after denervating the liver
14	Crural	2	350	430	+80	+23	Stimulation 3 hrs. 20 min. after denervating the liver
15	Sciatic	5	344	416	+72	+20	Stimulation 3 hrs. 10 min. after denervating the liver (see fig. 5)
16	Crural	4	476	512	+36	+7	Stimulation 3 hrs. 40 min. after denervating the liver
25	Sciatic	10	119	219	+100	+84	Liver denervated aseptically 35 days previously
30	Sciatic	15	146	207	+61	+41	Liver denervated aseptically 38 days previously
31	Sciatic	10	152	152			Liver denervated aseptically 34 days previously
36	Sciatic	5	164	167	+3	+1	Liver denervated and right adrenal removed aseptically 28 days previously
48	Sciatic	5	155	240	+85	+54	Liver denervated 28 days previously; also bilateral thyroparathyroidectomy and section of both cervical sympathetics 25 min. before stimulating
Average						+27	

* Experiment 11 was a rabbit; all the rest were cats.

moval of one adrenal and denervation of the other. In removing the adrenals, care was always taken to shell the gland out with as little damage to the splanchnic fibers as possible; to denervate an adrenal, the two splanchnics (major and minor) of the same side were cut just after their emergence from under the diaphragm and, in addition, any twigs connecting the adrenal with the upper lumbar ganglia were looked for and cut.

The data given in table 7 and illustrated, for two typical cases in figure 6, show that reflex stimulation of the adrenals is not an indispensable factor in the carbohydrate mobilization *effected by the strong afferent stimulation employed in these experiments;* this italicized qualification is

TABLE 7

The effect of afferent (crural, sciatic) stimulation on the blood sugar level of chloralose anesthetized animals after removal or denervation of the adrenals

EXPERIMENT NUMBER	NERVE STIMULATED	DURATION OF STIMULATION (MINUTES)	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR CHANGE IN PER CENT	REMARKS
			Before stimulation	After stimulation	Change		
12*	Crural	2	388	421	+33	+9	Both adrenals removed 2 hrs. 40 min. before the stimulation
20	Sciatic	3	190	191	+1		Both adrenals removed 1 hr. 40 min. before the stimulation
21	Sciatic	4	224	250	+26	+11	Right adrenal removed and left adrenal denervated 1 hr. before stimulation
22	Sciatic	4	344	338	−6	−2	Right adrenal removed and left adrenal denervated 2 hrs. 20 min. before stimulation
23	Sciatic	5	300	310	+10	+3	Both adrenals removed 1 hr. 15 min. before the stimulation
24	Sciatic	5	147	192	+45	+30	Both adrenals removed 1 hr. 20 min. before the stimulation (see fig. 6)
32	Sciatic	10	108	124	+16	+14	Right adrenal removed and left adrenal denervated 6 weeks previously
33a	Sciatic	5	114	155	+41	+36	Left adrenal removed and right adrenal denervated 5 weeks previously (see fig. 6)
33b	Sciatic	5	142	208	+66	+46	
34	Sciatic	30	87	104	+17	+19	Left adrenal removed and right adrenal denervated 5 weeks previously
Average						+18	

* Experiment 12 was a rabbit; the others were cats.

important in connection with certain evidence that will be cited in a paper to be published later. On the whole, however, the hyperglycemias of these cases are individually lower than those obtained in normal animals, and consistently enough so, to produce an average which is distinctly below the average for normal animals; and although, as was

said before, much importance cannot be attached to averages in this work, yet the following experiments lend further support to the conclusion that increases in the blood sugar value are less readily effected in the absence of adrenal coöperation.

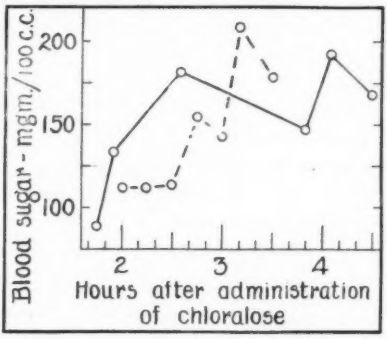

Fig. 6. A: Continuous line: Cat, experiment 24; 1:50–1:55, stimulation of left sciatic; 2:05–2:30, both adrenals removed by dorsal route; 4:00–4.05, stimulation of left sciatic. B: Broken line: Cat, experiment 33; on May 2, 1922, the left adrenal was removed and the right adrenal denervated aseptically under ether anesthesia. On June 9, 1922, as follows: 2:35–2:40, stimulation of left sciatic; 3:05–3:10, stimulation of left sciatic.

C. Reflex hyperglycemia after denervation of the liver and removal or denervation of the adrenals. If table 8 is inspected carefully it will be seen that the experiments of this class fall rather naturally, as judged by the results, into two groups: *1*, acute experiments in which all or part of the denervation was done just before the stimulation; and *2*, experiments in which the animal had had time to recover from an aseptic denervation operation which was done a few weeks previously. To the first group belong the following experiments, which may be summarized, with respect to the change in blood sugar value produced by the reflex (sciatic) stimulation, as follows:

EXPERIMENT NUMBER	BLOOD SUGAR CHANGE
	per cent
22	−3
27b	+19
28b	+12
30	+6
31	0
32	+1
33	−1
34	+49
53	+33
Average	+13

TABLE 8

The effect of afferent sciatic stimulation on the blood sugar level of chloralose-anesthetized cats after denervating the liver and removing or denervating the adrenals

EXPERIMENT NUMBER	DURATION OF STIMULATION (MINUTES)	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR CHANGE IN PER CENT	REMARKS
		Before stimulation	After stimulation	Change		
22	4	291	281	−10	−3	Right adrenal removed and left adrenal denervated 4 hrs. previously; liver denervated 1 hr. 10 min. previously
27a	10	94	106	+12	+13	Right adrenal removed, left adrenal and liver denervated 27 days previously
27b	10	161	192	+31	+19	Left adrenal removed 2 hrs. 30 min. previously
28a	10	67	78	+11	+16	Right adrenal removed, left adrenal and liver denervated 28 days previously
28b	10	156	175	+19	+12	Left adrenal removed 1 hr. 30 min. previously
30	10	229	243	+14	+6	Liver denervated 5 weeks previously; right adrenal removed and left adrenal denervated 2 hrs. 20 min. previously
31	10	153	153	0	0	Liver denervated 4 weeks previously; right adrenal removed and left adrenal denervated 1 hr. 40 min. previously
32	10	250	253	+3	+1	Right adrenal removed and left adrenal denervated 5 weeks previously; liver denervated 1 hr. 45 min. previously
33	10	219	217	−2	−1	Left adrenal removed and right adrenal denervated 5 weeks previously; liver denervated 2 hrs. 10 min. previously
34	10	243	363	+120	+49	Left adrenal removed and right adrenal denervated 5 weeks previously; liver denervated 2 hrs. 40 min. previously
44a	5	104	158	+54	+51	Right adrenal removed, left adrenal and liver denervated 6 weeks previously
44b	5	200	250	+50	+25	Same as 44a, but 3 hrs. later (see fig. 7)
45	10	94	118	+24	+25	Right adrenal removed, left adrenal and liver denervated 5 weeks previously
53	5	217	289	+72	+33	Liver denervated 4 weeks previously; both adrenals removed, by dorsal route, 1 hr. 35 min. previously

The following two experiments do not, strictly speaking, belong with the above and yet, in them, the liver and adrenals were denervated, in so far as splanchnic stimulation is concerned.

39	5	104	106	+2	+1	Right adrenal removed and splanchnics (major and minor) of both sides cut 4 weeks previously
40	5	106	118	+12	+11	Same as 39

Experiment 22 was really not negative; the blood sugar value was falling rapidly from a high level produced by the operation and the stimulation caused a check in the rate of fall, after the manner shown in figure 2; experiments 31, 32 and 33 really represent no change at all.

Although the average hyperglycemia of this series is not great (+ 13 per cent) and although there are conspicuous negative results, there are at the same time marked successes (exper. 27b, 34, and 53) which cannot be explained on the basis of mere chance. No special pleading for the negative results is to be made, but it seems only reasonable to recall what was said before, viz., there are so many reasons for expecting negative results in this work that clean-cut, positive results, such as experiments 34 and 53, should be worthy of the fullest credit. And the unexpected suggestion that reflex (sciatic) stimulation may still cause a marked hyperglycemia after the nervous connections of both the liver and adrenals have been destroyed, is strengthened by the other experiments of this series, which were done on animals that had fully recovered (insofar as weight and condition of nutrition are concerned) from an operation performed a few weeks previously:

EXPERIMENT NUMBER	BLOOD SUGAR CHANGE
	per cent
27a	+13
28a	+16
39	+1
40	+11
44a	+51 see fig. 7
44b	+25
45	+25
Average	+20

These figures are uniformly higher than those just previously given and the two series, taken together, leave no doubt that reflex (sciatic) stimulation can cause an increase in the blood sugar value even after the liver has been denervated and the adrenals have been removed or denervated.

Experiments 27b, 28b and 53 are especially interesting because they show that a reflex hyperglycemia may be produced after the liver has been denervated and both adrenals have been *completely removed;* in these cases the hepatic nerves had been cut 27 and 28 days before the experiment, but experiment 45, which was cited at the beginning of this section, shows that there is no reason to believe that these nerves regenerate in even a somewhat longer time than this; and, finally, the possibility of explaining the results as due to the activity of accessory adrenal tissue seems improbable on the basis of what we know in regard to it.

In conclusion, it seems worth noticing that the hyperglycemias produced after denervation of both liver and adrenals are more nearly of the order of magnitude of those produced after denervation of the adrenals alone (table 7) than of those produced after denervation of the liver alone (table 6); i.e., the result of this double operation is apparently due, almost entirely, to the removal or denervation of the adrenals; and the additional denervation of the liver seems, again, to have relatively little effect on the result.

2. THE RÔLE OF RESPIRATORY DISTURBANCES, AS JUDGED BY THE EFFECT OF CURARE. The preceding sections of this paper have shown that the hyperglycemia produced by afferent sciatic or crural stimulation seems to be in no way dependent upon the excitation of gluco-secretory

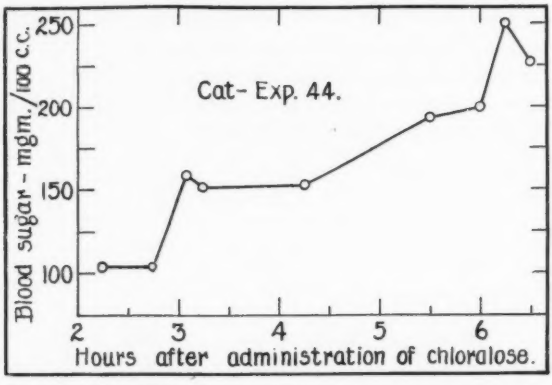

Fig. 7. On August 12, 1922: the right adrenal was removed and the liver and left adrenal denervated. On October 2, 1922: the left sciatic was stimulated centrally at 3:00–3:05 and again at 6:10–6:15 (see table 8).

nerves to the liver; neither does this form of hyperglycemia seem to be ultimately dependent upon an increased (reflexly excited) activity of the adrenals—although it is on the average somewhat greater when the adrenal mechanism is intact, than after removal or denervation of the glands. The problem still remains, therefore, as to the nature of the underlying mechanism whereby this increase in blood sugar concentration is effected.

The work of Henderson and Underhill (15) suggests that respiratory disturbances, with their resulting effect upon the acid-base equilibrium of the blood may be significant in the causation of many of the experimental hyperglycemias. Such stimulation as was used in this work produces great respiratory disturbance in an animal under chloralose anesthesia; and the predominating effect is undoubtedly an overven-

tilation during the application of the stimulus with, perhaps, a tendency toward acapnia. A similar situation, resulting from piqûre, was met by Henderson and Underhill by having their animals breathe an atmosphere containing an excess amount of carbon dioxide. In this present work, on the other hand, the possibility of respiratory disturbances was completely eliminated by the use of curare and continuous, uniform artificial respiration.

The use of curare was resorted to with a full realization of the hazard attendant upon its employment in such work as this. In the first place the available evidence seemed to show that curare might, of itself, cause a hyperglycemia. The literature on this subject has been reviewed by Macleod (16), but neither this review, nor Macleod's own work, nor a few further references not given by him, or appearing later (17), fully decide the question. It may in fairness be said, however, that the preponderance of opinion seems to be that if there is sufficient hepatic glycogen, curare will cause pronounced hyperglycemia, especially if there is any asphyxia; and there is some evidence that the drug has a specific hyperglycogenolytic effect, independently of any asphyxia it may cause. An opportunity existed, therefore, to supply new data which might help toward a decision in this matter.

But whether curare, of itself, causes any hyperglycemia or not, there is evidence of another kind to indicate that the results of its employment in this particular kind of work might be especially liable to misinterpretation. Curare not only paralyzes skeletal motor nerves, but it also, in sufficient dosage, paralyzes the preganglionic fibers of the autonomic system (18), (19). Any nervous mechanism regulating carbohydrate mobilization is hardly liable, therefore, to escape paralysis; and negative results from afferent stimulation would seem to be all that could be expected. Fortunately, however, curare does not act on all types of innervation alike; although it may be too soon, in view of the difference of opinion which seems to exist, to say in what order the curare paralysis does progress, it nevertheless is definitely established that the effect on various processes depends on the dose and the method of and time since administration. The possibility existed, therefore, that a dose that would paralyze the motor nerves to the respiratory muscles might not affect the nervous regulation of carbohydrate mobilization; and since this possibility had never been studied, results of any kind would naturally be of interest from this point of view. On the other hand, positive results (a hyperglycemia as the result of afferent stimulation) would have unusual interest in connection with the general problem of reflex hyperglycemia and would obviously have more than even the usual significance as compared with negative results; i.e., a negative result need not mean anything at all as to the rôle of respiratory disturbances

in this form of hyperglycemia, whereas a positive result would mean, unmistakably, that the reflex hyperglycemia is not due entirely to respiratory disturbances when they do occur.

Cats were used for all of these experiments. The anesthetization and all other details connected with the work, which were not new, were done as previously described. The curare was always freshly made up for every experiment, 2 cc. of 1 per cent solution, in distilled water, being prepared only an hour or two before it was needed. In almost all cases, 1 cc. of this 1 per cent solution was given intravenously (femoral vein) per cat. Artificial respiration was begun immediately after the injection and continued uniformly until the end of the experiment. The stimulation was done at varying intervals after the curare injection in the hope of finding an optimum time for the glycemic reaction while the respiratory nerves remained paralyzed. And it is worth while mentioning that during the stimulation careful notice was taken of the respiratory and other skeletal musculature; no animal showed the slightest trace of respiratory disturbance; often, however, the sciatic nerve was not damaged peripheral to the electrodes in these experiments and sometimes, but only rarely, the toes would give a slight twitch when a 5-second interval of stimulation began. There was something similar to a Wedensky effect, however, because the toes never went into a tetanus, i.e., even this movement, when it did occur, was neither vigorous nor prolonged; no other movement in the leg, or in the rest of the body, was ever observed in any of the experiments reported here.

In three of the following experiments (54, 58, 59) blood pressure records, which were taken at the end of the experiment and while the respiratory paralysis prevailed, showed pronounced pressor reactions (see the results of sciatic stimulation in the table on p. 655); this, however, does not indicate in what condition this portion of the autonomic system might have been half an hour or more previously, when the stimulations were made to test the glycemic response; and blood pressure records were never taken until the experiment was otherwise over, because the manipulations involved constituted just one more disturbance which was not indispensable and which it was thought had best be avoided.

A. The effect of curare on the blood sugar value. In accordance with the practice employed in this work, of determining the trend of the blood sugar changes before applying any special experimental test, two or more blood samples were usually taken before and after the curare injection in order to be able to evaluate the effect of the injection itself. The data thus accumulated are shown in table 9; inspection of this table shows that the effect of curare (with continuous, uniform artificial respiration) on the blood sugar level varies from one animal to

another; there is a slowly developing hyperglycemia, following the injection, in about one-half of the cases; in the remaining cases no change, or a slight decrease in the blood sugar value takes place. The statements in the literature as to the inconstancy of the curare effect are, therefore, confirmed. There are no new data at hand to explain this variability, but it is not impossible that it may be due to the nature of the artificial respiration rather than to the curare, *per se*. There is no doubt that asphyxia will cause a hyperglycemia and if acapnia has

TABLE 9

The effect of curare—1 cc. of 1 per cent, per animal—on the blood sugar value of chloralose-anesthetized cats, with continuous, uniform artificial respiration

	EXPERIMENT NUMBER	WEIGHT OF CAT (KGM.)	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.															EFFECT OF THE CURARE ON THE BLOOD SUGAR VALUE
			Before curare injection	After curare injection														
				Time after injection in minutes														
				10	15	25	30	35	40	45	50	55	60	65	70	75	90	110	
Normal cats	37	3.30	153		160		165											+
	42	?	172				151						144				135	−
	49	2.20	180				219			251								+
	50	2.65	104				131			178			176					+
	51	2.50	253	256		253			246									0
	52	?	122			150			200									+
	54	2.05	150								190			200				+
	55	2.45	130		150		153			162								+
	56	3.00	206		198		196			196								0
	57	?	152		150			155				163			168			0
	63	2.45	243						212							169	163	142 −
Liver and adrenals denervated	58	3.25	152		153		162			170								+
	59	3.72	?		229		235						246					+?
	64	3.80	?	108			105									99		−?
	65	2.80	?						166			155						−?
	66	3.20	?						153				140					−?
	67	3.90	?			153					175							+?
	68	3.10	193			177							166					−

The first 11 experiments in this table (37 to 63 inclusive) were performed on normal animals; the remaining experiments (58, 59 and 64 to 68 inclusive) were performed on animals having the liver denervated and the adrenals out or denervated (the complete data in regard to the operations in experiments 58, 59, 64 and 65 are given in table 13; experiments 66, 67 and 68 were similar to 65); in all but two of these latter experiments the blood sugar value was not determined before the curare injection; but the general trend of the values after the injection is probably a reliable indication of the effect the curare had; nevertheless a question mark is appropriately placed after the supposed sign of the reaction.

the same effect it can be seen that in order for the artificial respiration to be just adequate it would have to be adjusted both as to rate and volume for each individual case. The means for doing this were not available for these experiments and it is a precaution that does not appear to have been taken in any previous work on this subject. The artificial respiration in these experiments was carried out at a rate between 20 and 30 per minute and was of a volume that seemed to distend the chest normally; this was adequate to keep the blood well aerated. But the crudeness of such an approximation to what might have been the real need of the animal at the time is too obvious to need emphasis. Likewise, there may be no reason for doubting that in those cases that show little or no change in blood sugar value the artificial respiration happened, by chance, to be somewhat closely approximated to the real need of the organism at the time; in the other cases an overventilation is probably more to be suspected than an asphyxia, but either might account for the slowly developing hyperglycemias that were observed.

One further point worth mentioning here, because of its importance in the following work, is that this hyperglycemia, when it does occur, is usually of gradual and uniform development; so it is not liable to mask the abrupt changes produced by stimulation.

B. The effect of curare on the reflex hyperglycemia. *a.* Normal animals. Table 10 contains a record of the experiments that come under this heading; the results show unmistakably that curare diminishes the average intensity of the hyperglycemia produced by reflex sciatic stimulation. It is not easy to be certain, however, as to the cause of this effect: is it due *1*, to the elimination of respiratory disturbances, which is the point of special interest in the present work; or *2*, to the paralysis of some peripheral nervous mechanism involved in carbohydrate mobilization; or *3*, to both acting together? One thing may be definitely stated, again, in regard to these experiments, however, and that is, the stimulation caused no respiratory disturbance in any of them; in other words, the very respectable hyperglycemias produced in experiments 37, 43 and 55 are in no way attributable to this factor. On an absolute basis, this is a small percentage of positive responses; but when the hazards connected with the use of curare, which have been thoroughly gone into, are kept in mind, a low frequency of positive responses is not unexpected; and these positive responses may, therefore, possess a significance quite disproportionate to their frequency.

The one conclusion that may be made with certainty from these experiments is that the reflex hyperglycemia is not due entirely to respiratory disturbances; whether such disturbances, with their sequence of change in the acid-base equilibrium of the body fluids, play any rôle

in this form of blood sugar concentration is not so easily concluded from the data. I personally believe, from the evidence of the positive results of average intensity in this series, that this factor probably plays a very small part, if any at all, in this reaction. This is not to deny that respiratory disturbance may alter the blood sugar value; but it would seem only logical to differentiate between the effects of long-continued asphyxia or acapnia—such as might result from prolonged artificial respiration of an inadequate character—and such temporary and transient disturbances as might be caused by the brief periods of stimulation employed in most of this work. Very rarely was the total period of stimulation longer than 10 minutes (usually 5 minutes) and even then

TABLE 10

The effect of afferent sciatic stimulation, for 5 minutes, on the blood sugar value of normal cats under chloralose anesthesia, curare (1 cc. of 1 per cent per animal) and continuous, uniform artificial respiration

EXPERIMENT NUMBER	WEIGHT OF CAT (KGM.)	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR CHANGE IN PER CENT	TIME BETWEEN CURARE INJECTION AND STIMULATION (MINUTES)
		Before stimulation	After stimulation	Change		
37	3.30	165	192	+27	+16	35
42	?	135	143	+8	+5	75
43	?	263	336	+73	+27	25
49	2.20	251	285	+34	+13	30
50	2.65	176	180	+4	+2	35
51	2.50	246	268	+22	+9	40
52	?	200	202	+2	+1	35
54	2.05	200	198	−2	−1	30
55	2.45	162	294	+132	+81	50
56	3.00	196	212	+16	+9	45
57	?	168	185	+17	+10	60
Average					+15	

it was of an interrupted character (on 5 seconds and off 5 seconds, alternately), so that the period during which the stimulation was actually capable of affecting the respiration was only half of the total recorded duration.

b. After denervation of the liver and removal or denervation of the adrenals (and total thyroparathyroidectomy and denervation of the pancreas and hypophysis). The experiments of this series are too few to be altogether conclusive, but it is worth while to include here what has been done in this direction. See table 11, which shows that reflex stimulation may still produce an increase in the blood sugar concentration even under curare and the conditions defined in the title to this section. No emphasis is to be placed on the additional operations

practised in some of these cases, because the experiments are too few to permit any generalization; but the thyroparathyroidectomy was performed so as to exclude any possible reflex excitation of these glands; the cervical sympathetics were cut in order to denervate the hypohysis (?); and the nerves along the hepatoduodenal and pancreatoduodenal arteries were cut so as to denervate the pancreas as well as the liver;

TABLE 11

The effect of afferent sciatic stimulation, on the blood sugar value of cats under chloralose anesthesia, curare (1 cc. of 1 per cent per animal) and continuous, uniform artificial respiration; and after denervation of the liver and denervation or removal of the adrenals

EXPERIMENT NUMBER	WEIGHT (KGM.)	DURATION OF STIMULATION (MINUTES)	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR INCREASE IN PER CENT	REMARKS
			Before stimulation	After stimulation	Increase		
58	3.25	5	170	202	32	18	Right adrenal removed and left adrenal and liver denervated 4 weeks previously
59	3.72	5	246	266	20	8	Right adrenal removed and liver denervated 3 weeks previously; left adrenal removed by dorsal route 2 hrs. 30 min. before stimulation
64	3.80	10	99	116	17	17	Right adrenal removed and left adrenal and liver denervated 4 hrs. before stimulation
65	2.8	5	155	163	8	5	Right adrenal removed and left adrenal and liver denervated 4 hrs. 10 min. before stimulation

In experiments 59, 64, 65 the thyroids and parathyroids were removed and both cervical sympathetics cut low in the neck at the time of the experiment and preceding the stimulation.

In experiment 64 the liver was denervated by cutting the nerves along the hepatoduodenal artery rather than in the hepatic pedicle.

In experiment 65 the liver was denervated as in experiment 64 and, in addition, the nerves accompanying the pancreato-duodenal artery were cut.

the experiments may be taken to indicate a strong probability that none of these glands are indispensable for this form of hyperglycemia.

Experiments 58 and 64 are especially interesting when considered in connection with the previous work. It was shown in the second section of this paper (see table 7) that removal or denervation of the adrenals reduced the average reflex hyperglycemia to 18 per cent; these two

experiments (58 and 64) indicate that this residual hyperglycemia, which occurs without adrenal coöperation, is not due to respiratory disturbances.

It may be significant that the *average* hyperglycemia produced by afferent sciatic stimulation, in curarized cats, is of the same order of magnitude (+ 18 per cent ±) as the hyperglycemia, produced in the same way, in cats whose adrenals have been immobilized (both out, or one removed and the other denervated); for Stewart and Rogoff (19) claim that the preganglionic fibers to the adrenals are among the first components of the autonomic system to be paralyzed by curare. In other words, the usual reduction of the hyperglycemia under curare may lend further support to the evidence already presented, that the adrenals do, normally, contribute to the magnitude of this form of hyperglycemia. If this is the true interpretation of the curare effect, it leaves still less of the usual hyperglycemia in normal animals to be explained as due to respiratory disturbance.

REFLEX HYPERGLYCEMIA FROM CENTRAL VAGUS STIMULATION

The cause of the hyperglycemia which results from stimulation of the central end of the vagus nerve has been variously explained. Bernard believed that the result depended upon stimulation of the "sugar center" and was, therefore, ultimately analogous to piqûre (20, p. 392); he thought that the respiratory disturbance caused by such stimulation had no part in producing the result (21, ii, p. 446). Bang, however, has made a distinction between the mechanism of piqûre and that of central vagus stimulation (22); according to him, piqûre affects principally hepatic glycogen, whereas central vagus stimulation affects, chiefly, muscle glycogen. In a later paper (23), however, he reported that proper anesthesia prevented the usual hyperglycemia from piqûre, central vagus stimulation and some other procedures, and seemed to conclude that these varieties of hyperglycemia are alike in that they are but different forms of "psychic-hyperglycemia."

Starkenstein (24) and Rossi (25) are agreed that the hyperglycemic effect of central vagus stimulation is independent of the adrenals.

First Laffont (20, p. 392) and later Macleod came to the conclusion that asphyxia is the ultimate cause of this form of hyperglycemia. The latter says (20, p. 406), "Stimulation of the central ends of the vagus nerves in the neck (both nerves being cut) causes hyperglycemia when no precautions are taken against asphyxia. When asphyxia is carefully guarded against (by free administration of oxygen and artificial respiration), stimulation of the central end of the vagus nerve in the neck does not cause hyperglycemia. There is no evidence that stimula-

tion of the central end of the vagus nerve can reflexly produce hyperglycemia."

The curarized cats, under uniform artificial respiration, that were used in the preceding experiments, seemed to offer good material on which to test this question in a manner that does not appear to have been previously tried. The general conditions of these experiments were similar to the previous ones. For these particular experiments the vagi (vagus-depressor trunks in the cat) were usually dissected out and separated from the cervical sympathetic trunks only just before they were to be stimulated. The vagi were ligated, cut distal to the ligature and the central ends of the two nerves stimulated with a tetanizing current (from the secondary coil of a Harvard inductorium; 4.5 volts, 0.3 ampere in the primary circuit; coil distance 4 or 6 cm.) for 30 seconds, alternately, for 5 minutes, i.e., each nerve was stimulated $2\frac{1}{2}$ minutes in all. These vagus stimulations were all done on cats that had just previously (within 20 to 30 minutes) had a reflex hyperglycemia produced by stimulation of the central end of the sciatic, i.e., the vagus effect is often more or less superimposed upon a previous hyperglycemia and, therefore, the percentage rise in blood sugar value induced by the vagus stimulation may not be as great as if the blood sugar level had not previously been disturbed. The vagus stimulation was not begun, however, until long enough after the previous sciatic stimulation to insure that the hyperglycemia caused by the latter would be on the decline; this was determined by taking one or more blood samples between the peak of the sciatic hyperglycemia and the beginning of vagus stimulation (see figs. 8 and 9); in this way there is no difficulty in determining the effect of the vagus stimulation, itself, on the blood sugar curve.

Tables 12 and 13 give the results of these experiments on central vagus stimulation, in which respiratory disturbances were excluded by curare paralysis and artificial respiration; figures 8 and 9 illustrate typical cases and with their legends give an idea of the conduct of the experiments. The hyperglycemias observed in these experiments, although not very intense on the average, are unmistakably distinct and are, in individual cases, as great as the reflex hyperglycemias resulting from sciatic or crural stimulation. It should be noticed that in experiments 65, 66 and 67 of table 13, not only was one adrenal out and the other adrenal and the liver denervated, but an effort was made to isolate every gland that is known or has been supposed to play a part in blood sugar regulation—i.e., the thyroids and parathyroids were completely removed; the hypophysis was denervated by sectioning the cervical sympathetics (?); and the pancreas was denervated by sectioning the nerves along the hepatoduodenal and pancreatoduodenal arteries. However effective

TABLE 12

The effect of afferent vagus (vagus-depressor trunk) stimulation for 5 minutes on the blood sugar value of normal, chloralose-anesthetized cats, under curare (1 cc. of 1 per cent per animal) and continuous uniform artificial respiration

EXPERIMENT NUMBER	WEIGHT OF CAT (KGM.)	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR INCREASE IN PER CENT	TIME OF STIMULATION AFTER INJECTION OF CURARE (MINUTES)
		Before stimulation	After stimulation	Increase		
49	2.20	268	320	52	19	105
50	2.65	178	198	20	11	75
52	?	198	217	19	9	75
54	2.05	198	222	24	12	90
55	2.45	294	417	123	41	70
56	3.00	188	210	22	11	70
61	2.20	336	373	37	11	25
Average					16	

TABLE 13

The effect of afferent vagus (vagus-depressor trunk) stimulation, for 5 minutes, on the blood sugar value of cats, under chloralose anesthesia, curare (1 cc. of 1 per cent per animal) and artificial respiration; after denervation of the liver and removal or denervation of the adrenals

EXPERIMENT NUMBER	WEIGHT OF CAT (KGM.)	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.			BLOOD SUGAR INCREASE IN PER CENT	TIME OF STIMULATION AFTER INJECTION OF CURARE (MINUTES)	REMARKS
		Before stimulation	After stimulation	Increase			
58	3.25	227	333	106	46	105	Right adrenal removed; left adrenal and liver denervated 4 weeks previously
59	3.72	253	266	13	5	115	Right adrenal removed and liver denervated 3 weeks previously; laparotomy; removal of left adrenal; bilateral thyro-parathyroidectomy and section of both cervical sympathetics 4 hours before stimulation
64	3.80	108	123	15	13	120	See the legend of figure 9
65	2.80	153	185	32	20	105	Same operation as 64; in addition the nerves along the pancreaticoduodenal artery cut (pancreas completely denervated?); 5 hours before stimulation
66	3.20	126	131	5	4	90	Same operation as 65; 3 hours before stimulation
67	3.90	194	250	56	28	85	Same operation as 65 and 66; 5 hrs. 30 min. before stimulation
Average					19		

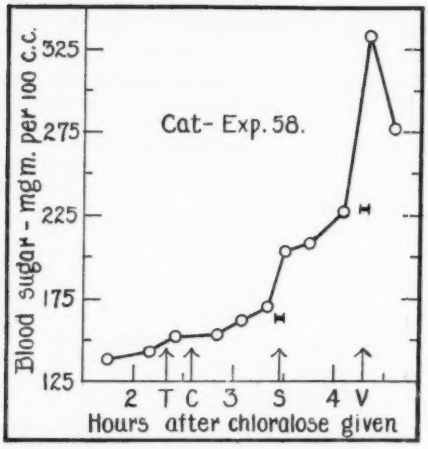

Fig. 8. Cat; ♂; November 17, 1922; weight, 2.72 kgm.; under ether anesthesia the following operation was performed aseptically: laparotomy; right adrenal removed; left adrenal and liver denervated.

December 18, 1922; weight, 3.25 kgm. 8:45 a.m. 0.3 gram chloralose in 25 cc. milk by stomach tube; 10:00 a.m. tied down and cannula in femoral artery; 11:00 a.m. insertion of tracheal cannula, *T*; 11:15 a.m. 1 cc. 1 per cent curare in femoral vein and artificial respiration begun and continued to end of experiment, *C;* 12:05–12:10 p.m. stimulated central end of left sciatic, *S;* blood sugar value went from 170 to 202 mgm. per 100 cc. (+18 per cent); 1:00–1:05 p.m. stimulated central ends of vagi in neck for 5 minutes, *V;* blood sugar value raised from 227 to 333 mgm. per 100 cc. (+46 per cent).

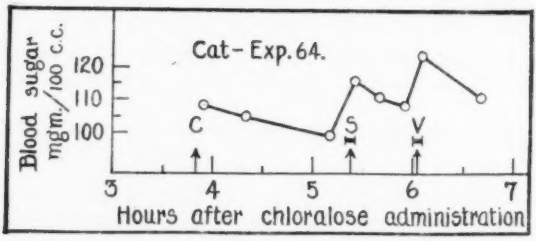

Fig. 9. Cat; ♂; weight, 3.8 kgm.; at 9:15 a.m. (March 29, 1923) the cat was quickly and lightly etherized, tied down and 20 cc. 1 per cent chloralose, in mammalian Ringer, injected in the femoral vein (9:30); ether off; laparotomy; removal of right adrenal; denervation of the left adrenal; liver denervated by cutting the nerves along the hepatico-duodenal artery (which also partially denervates the pancreas); abdomen sewed up; bilateral thyro-parathyroidectomy and section of both cervical sympathetic trunks low in the neck. Operation over by 10:30.

At 1:20 (*C* in the figure) 1 cc. of 1 per cent curare injected in femoral vein; artificial respiration begun immediately and continued to the end. At *S* and *V* the central ends of the left sciatic and both vagus nerves were stimulated for 5 minutes, respectively; at *S* the blood sugar value went from 99 to 116 mgm. per 100 cc. (+17 per cent); at *V* the blood sugar value went from 108 to 123 mgm. per 100 cc. (+13 per cent).

or significant these attempted, additional denervations may have been, there is no doubt that this form of hyperglycemia, like that resulting from afferent sciatic stimulation, is independent of the activity of gluco-secretory nerves to the liver and, as was previously observed by Starkenstein and by Rossi, of a reflexly stimulated adrenal activity.

If one examines Macleod's data as to the effect of central vagus stimulation, rather than his conclusions therefrom, it will be seen that there is no real conflict between the results reported by him and those given here; in only a few cases did he determine the blood sugar value before the stimulation; but in every case in which he did (except one where artificial respiration was *not* given) the blood sugar value was greater after the stimulation than before, whether artificial respiration was given or not. The blood sugar values were uniformly higher and the glycosuria much more pronounced in those cases where artificial respiration was not employed than in those where it was; so Macleod ascribed the increase in the latter case to the anesthetic and other factors inherent in the experiment. It is undoubtedly true that the anesthetic in both classes of animals and the asphyxia in the unventilated animals would contribute to the total hyperglycemia; but Macleod's method did not enable him to separate out the vagus effect from the total, complicated situation.

DISCUSSION

The results reported in this paper show that reflex vagus, crural or sciatic stimulation may cause an increase in the concentration of sugar in the blood. The increase which takes place as a result of afferent crural or sciatic stimulation is not affected by sectioning the nerves to the liver; it is diminished (on the average, from 31 per cent to 18 or 20 per cent, in cats) by immobilization of the adrenals (both out, or one out and the other denervated) and by curare (with continuous, uniform artificial respiration). If, as seems probable, the diminished effect under curare may be due to paralysis of some motor mechanism other than the respiratory (e.g., the preganglionic fibers to the adrenals) it would seem that respiratory disturbances (acapnia) do not contribute to the magnitude of the response under normal conditions. At all events, a residual hyperglycemia of 18 to 20 per cent remains to be accounted for after the nerves to the liver have been sectioned, the adrenals immobilized, and respiratory disturbances eliminated.

Vagus stimulation was always done under curare and the result is not affected by sectioning the nerves to the liver, nor by immobilizing the adrenals (by removal or denervation).

A few experiments involving both vagus and sciatic stimulation, under curare, indicate that neither the pancreas, thyroid, parathyroid or

hypophysis (?) are involved in the development of this residual hyperglycemia of 18 to 20 per cent; and the inference is that they play no part in the somewhat greater reaction to sciatic or crural stimulation (+ 31 per cent) which occurs if the adrenals are intact or the animal is not curarized.

If, therefore, the blood sugar concentration can be increased as much as 18 or 20 per cent under conditions in which *1*, respiratory disturbances, or *2*, reflex stimulation of gluco-secretory nerves to the liver, or reflexly stimulated activity of the *3*, adrenals, *4*, pancreas, *5*, thyroids, *6*, parathyroids, and *7*, hypophysis are impossible, the cause of this concentration of the blood sugar must be sought in some other result of such reflex stimulation.

Lamson's work (26) on the increased concentration of red corpuscles in the blood, as a result of certain forms of stimulation, seemed at first sight to be a more or less related phenomenon. But the relationship is only superficial; for Lamson found that the vasoconstrictor effect of adrenin on the hepatic capillaries was the ultimate cause of the reaction studied by him; this resulted in a partial obstruction of the blood flow through the liver, and as a consequence of the rise of hepatic blood pressure, the plasma was filtered into the liver lymphatics; there seemed to be some absolute increase in the concentration of the corpuscles, also, but the above mechanism accounted for most of the change. This reaction did not take place, in response to afferent stimulation and fright after removal of the adrenals; nor did it occur in rabbits, whose liver capillaries do not react to adrenalin. Even if it is assumed that the blood sugar molecules, on account of their own size or by virtue of being combined with a protein, might be concentrated by filtration in this way, still such a mechanism can seem to have no relation to the cause of the reflex hyperglycemia, for this does occur in rabbits and it also occurs in cats after the adrenals have been immobilized. Therefore, unless a concentration of the blood may be effected in some, as yet, unknown way by means of afferent stimulation, the reflex hyperglycemia must be looked upon as the result of an active mobilization of the carbohydrate stores.

The question, then, arises as to the source of the mobilized carbohydrate; if it is muscle glycogen, the reaction might be explained as the result of the action of gluco-secretory nerves to the muscle; if the liver is the source of the mobilized carbohydrate, the preceding evidence seems to have eliminated every other possible cause of hepatic glycogenolysis except alterations of the blood flow through that organ; this is the explanation of hepatic hyper-glycogenolysis that has been defended by Wertheimer and Battez (14).

If alterations in blood flow (pressure) are to be held responsible for this type of carbohydrate mobilization, it will be necessary to explain how such apparently diametrically opposite effects as are produced on the blood pressure by afferent sciatic and vagus stimulation, respectively, can have such identical effects on the blood sugar mobilization. Blood pressure records were taken in only three of the experiments previously cited (nos. 54, 58 and 59) and then at the end of the experiment, after the blood sampling for sugar determination was completed. Each of these cats had been curarized and the curare effect still prevailed (insofar as the paralysis of the respiratory nerves was concerned) at the time the blood pressure determinations were made; the blood pressure reactions were as follows:

EXPERIMENT NUMBER	BLOOD PRESSURE IN MM. Hg		NERVE STIMULATION
	Before stimulation	After stimulation	
54	130	38	Vagus
	111	160	Sciatic
58	136	54	Vagus
	182	214	Sciatic
59	104	30	Vagus
	106	146	Sciatic

As was said, these blood pressure records were taken after the samples for sugar determination had been obtained and this might be thought to make them poor indices of what happened when the nerves were stimulated (half an hour and more previously) to produce the hyperglycemia. A fairly reliable indication that blood pressure changes of this same sort occurred uniformly—no matter how long (within the limits used in this work) the cat had been anesthetized or curarized before the stimulation—is seen in the fact that the cannula in the femoral artery always filled faster after sciatic or crural stimulation, and more slowly after vagus stimulation, than normally. In fact, in two cases (which have not been used simply because of this result) central vagus stimulation was fatal. There can be no doubt, therefore, that such stimulation as was employed in this work caused marked alterations in the blood pressure and necessarily marked alterations in the blood flow throughout the organs of the body. It may be that either a positive or negative variation from the normal blood pressure can cause glycogenolysis in the carbohydrate depots by two different mechanisms; or, it is not impossible that the vasoconstriction associated with sciatic stimulation might have the same ultimate effect (asphyxia) as the sluggish flow resulting from vagus stimulation.

None of these suggestions are to be taken seriously in their present unsupported form. They are simply mentioned in order to show the direction in which the results of these experiments seem to lead.

SUMMARY

1. Operations, especially those within the abdomen (removal of adrenals; dissection of hepatic nerves), cause hyperglycemia; but, most significant for such work as this, these disturbances of the blood sugar concentration constitute an abnormal condition out of which it is very difficult to obtain a normal response to other hyperglycemic procedures.

2. The stimulation of the central end of the crural or sciatic nerve produces a prompt increase in the blood sugar value of normal rabbits and cats under chloralose anesthesia.

3. This reflex hyperglycemia varies greatly in intensity from one animal to another even under as standard conditions as it is possible to maintain and reproduce.

4. After denervating the liver, stimulation of the central end of the sciatic or crural nerve has practically as great an effect in raising the blood sugar level as in normal animals.

5. It makes no difference whether both adrenals are removed in an acute experiment, or whether one adrenal is removed and the other denervated; in either case the hyperglycemia produced by reflex (sciatic, crural) stimulation, although it may be of considerable degree, is on the *average* less than that produced in normal animals.

6. The effect of reflex (sciatic) stimulation after denervating the liver and also removing or denervating the adrenals is of the same order of magnitude as that observed after denervation or removal of the adrenals alone.

7. The effect of curare (with continuous, uniform artificial respiration) on the blood sugar level is variable, in that a slowly developing hyperglycemia follows its injection in about one-half of the cases, whereas no change or a slight decrease in the blood sugar value is the result in others. It is suggested that the hyperglycemia, when it does occur, may not be due to the curare, *per se*, but to the unphysiological character of the artificial respiration in relation to the real respiratory need of the organism at the time.

8. Curarization (with continuous, uniform artificial respiration) does not prevent the development of a reflex hyperglycemia in normal cats, although the average increase in the blood sugar concentration is much reduced under these circumstances; nevertheless, individual hyperglycemias of sufficient intensity occur often enough (in the total absence of respiratory disturbances) to suggest that the usual diminution of the response, by the curare, may be due to some other effect of

the curare than its elimination of respiratory disturbances; it is suggested that the diminution in response, when it does occur, may be due to the paralysis of the preganglionic fibers to the adrenals by the curare.

9. Two experiments are recorded in which the blood sugar concentration was increased 18 and 17 per cent, respectively, by afferent sciatic stimulation, after denervation of the liver and adrenals and while the animals were curarized and under uniform, continuous artificial respiration. Since an increase in the blood sugar concentration of 18 per cent is the average magnitude of the hyperglycemia produced in similarly denervated animals, in which respiratory disturbances did occur, it seems again very doubtful whether these disturbances are of much importance in the production of this form of hyperglycemia.

10. Stimulation of the central ends of the vagi (vagus-depressor trunk) in the cat causes an immediate increase in the concentration of sugar in the blood, in the entire absence of any respiratory disturbance (i.e., in cats that are curarized and are being uniformly and continuously ventilated by artificial respiration).

11. This hyperglycemia, like that resulting from reflex crural or sciatic stimulation, takes place, with no apparent diminution of average intensity, even after the adrenals have been immobilized (both out or one out and the other denervated), the nerves to the liver, pancreas, and hypophysis (?) cut and the thyroid-parathyroid apparatus completely removed.

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FURTHER EVIDENCE REGARDING THE RELATIONSHIP OF THE ADRENALS TO THE AMOUNT OF SUGAR IN THE BLOOD

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It has previously been shown (1) that it is possible, in individual cases and by means of strong sciatic or crural stimulation, to produce as intense a hyperglycemia, after removal or denervation of the adrenals, as usually occurs in normal animals. But the evidence also showed that this reflex hyperglycemia is, on the average, somewhat less when adrenal participation is impossible than it is in normal animals. The conclusion was, therefore, drawn that the adrenals do, normally, play a part in the carbohydrate mobilization resulting from reflex stimulation.

The purpose of this paper is to present further evidence showing that the adrenals are related to carbohydrate mobilization. This evidence was derived from the animals that served for the experiments recorded in the previous paper, but instead of having to do with their blood sugar values after strong sensory stimulation, it is designed to call attention to the relationship between the adrenals and the amount of sugar in the blood under standard laboratory conditions.

In order to understand the meaning of this new evidence it is necessary to have in mind the treatment which the animals (cats in all of this work) received. In all cases where the liver or adrenals or both were denervated, the operation was performed aseptically, under ether anesthesia (1, pp. 633–637); sufficient time was then allowed for recovery (never less than three weeks) to a good nutritive condition. There was no apparent difference between the behavior or health of the operated animals at the time they were used for the experiment and the normal animals; except that, on account of the care and food they had received, the operated animals invariably weighed slightly more at the time of the experiment than they had when operated on a few weeks previously.

The effort was therefore made to have all of the cats—whether normal or previously operated on—in a good condition of nutrition

at the time of the experiment. Furthermore, the treatment they received in preparation for an experiment was as standardized as possible; the cat was carried from the animal pens down one flight of stairs to the laboratory, as gently and with as little frightening as possible; it was then turned loose in the laboratory, allowed to drink its dose of chloralose in milk and left entirely to itself until anesthesia was complete—usually a matter of one and a half or two hours; it was then tied down, a cannula was inserted in the femoral artery and the first or "initial" blood sample taken. This is a brief recapitulation of the description given in greater detail previously (1, pp. 624), but it is important to emphasize again, and in this particular connection, the kind and uniformity of the treatment which all of these cats received.

The first point to which attention is to be called is the initial blood sugar values recorded in table 1. The average value for normal cats is 153 mgm. per 100 cc.; that for cats whose livers had been denervated is practically the same—152 mgm. per 100 cc.; whereas those cats whose adrenals had been immobilized (one out and the other denervated) have an average initial blood sugar value of only 99 mgm. per 100 cc. This evidence seems to show conclusively enough that there is an important relationship between the functional activity of the adrenal glands and the amount of sugar in the blood under even such apparently identical conditions as all of these animals were subjected to; and the further conclusion seems substantiated, the denervation of the liver, alone, has no significant result on the blood sugar level; nor does this operation, added to the immobilization of the adrenals, produce any additional effect.

Further evidence along these same lines and of an equally significant kind is also found in table 1. Not only are the initial blood sugar values lower, when the adrenals are immobilized, than they are in normal animals or in animals whose livers, alone, have been denervated; but so long as nothing drastic is done to the animal (no strong sensory stimulation, etc.) the blood sugar level remains lower and is less variable in the former than in the two latter classes. Thus, the third column of the table gives the blood sugar value of the second blood sample; the fourth column gives the time it was taken after the first; and the fifth column gives the difference in milligrams per 100 cc. between the sugar content of the first (initial) and the second samples. Here, again, the values for the normal animals and for those whose livers, alone, were denervated are of the same order of magnitude; on the other hand, both of these classes contrast strongly with those animals whose adrenals were immobilized and whose blood sugar values showed remarkably little change under the conditions of these experiments.

TABLE 1

EXPERIMENT NUMBER	INITIAL BLOOD SUGAR VALUES (MGM. PER 100 CC.)	SUGAR VALUE OF SECOND BLOOD SAMPLE (MGM. PER 100 CC.)	TIME FROM INITIAL TO SECOND SAMPLE (MINUTES)	DIFFERENCE BETWEEN INITIAL AND SECOND BLOOD SUGAR VALUES (MGM. PER 100 CC.)	REMARKS
39	95	104	15	+9	Liver and one adrenal denervated; other adrenal out
40	111	106	15	−5	Liver and one adrenal denervated; other adrenal out
46	117	116	15	−1	Liver and one adrenal denervated; other adrenal out
33	112	112	15	0	One adrenal out; the other denervated
27a	94	94	30	0	Liver and one adrenal denervated; other adrenal out
44a	104	104	30	0	Liver and one adrenal denervated; other adrenal out
32	114	108	30	−6	One adrenal out; the other denervated
34	86	86	30	0	One adrenal out; the other denervated
45	93	94	40	+1	Liver and one adrenal denervated; other adrenal out
28a	67	67	45	0	Liver and one adrenal denervated; other adrenal out
	99				Average
36	175	167	15	−8	Liver alone denervated
30	141	146	30	+5	Liver alone denervated
25	100	119	45	+19	Liver alone denervated
31	210	175	60	−35	Liver alone denervated
48	136				Liver alone denervated
	152				Average
37	108	104	15	−4	Normal cat
55	122	128	15	+6	Normal cat
43	157	195	20	+38	Normal cat
51	122	169	20	+47	Normal cat
22	205	212	30	+7	Normal cat
26	87	93	30	+6	Normal cat
35	168	183	30	+15	Normal cat
13	197	177	35	−20	Normal cat
20	147	151	45	+4	Normal cat

TABLE 1—*Concluded*

EXPERIMENT NUMBER	INITIAL BLOOD SUGAR VALUES (MGM. PER 100 CC.)	SUGAR VALUE OF SECOND BLOOD SAMPLE (MGM. PER 100 CC.)	TIME FROM INITIAL TO SECOND SAMPLE (MINUTES)	DIFFERENCE BETWEEN INITIAL AND SECOND BLOOD SUGAR VALUES (MGM. PER 100 CC.)	REMARKS
29	113	141	60	+28	Normal cat
21	145	114	65	−31	Normal cat
14	261				Normal cat
23	243				Normal cat
24	88				Normal cat
38	163				Normal cat
42	172				Normal cat
49	180				Normal cat
50	104				Normal cat
52	122				Normal cat
54	150				Normal cat
56	190				Normal cat
57	141				Normal cat
	153				Average

The question naturally arises as to why the immobilization of the adrenals results in such relatively low and stable blood sugar values. And—after it is again insisted that there was no difference in the handling and treatment of the animals at the time of the experiment, which would be sufficient to account for the difference in results—it may be suspected that the effect of such an operation is indirect and by virtue of interference with the normal carbohydrate metabolism. As was said before, the animals seemed to be in good condition, insofar as their weight and appearance were concerned; but it is to be regretted that the hepatic glycogen was not estimated in all these cases. This determination was made only in connection with a few of the later experiments and then only in one case where the adrenals had been previously immobilized; furthermore, in all of these cases the glycogen determination was made at the end of an experiment, after the animal had been anesthetized for several hours, had been curarized and had had hyperglycemias produced by sciatic or vagus stimulation. Yet the experiments were near enough alike so that the amount of glycogen left in the liver at the end of them may have some significance for purposes of rough comparison.

The figures, such as they are, follow:

EXPERIMENT NUMBER	REMARKS	HEPATIC GLYCOGEN
		per cent
50	After curare and sciatic stimulation; otherwise normal	0.40
51	After curare and sciatic stimulation; otherwise normal	2.10
54	After curare and sciatic stimulation; otherwise normal	0.84
56	After curare and sciatic stimulation; otherwise normal	1.59
53	Hepatic nerves cut 4 weeks previously; adrenals removed at time of experiment; sciatic stimulation	1.50
58	Right adrenal out and liver and left adrenal denervated 4 weeks previously; curare; sciatic and vagus stimulation	2.12

This single determination (exper. 58) is only of value as bearing out the general impression that the operated animals were, as a rule, in better nutritive condition when they were used for the experiment, after recovery, than they were originally, or than were the usual run of normal animals.

The possibility that the low and stable blood sugar values after immobilization of the adrenals is due to depleted carbohydrate stores, is further discredited by the pronounced hyperglycemias which have been produced, by different means, in many of the animals of table 1. Thus, in experiments 33, 34, 44 and 45, in spite of the fact that the blood sugar value was relatively low and remained so, under the general condition of the experiments, yet stimulation of the central end of the sciatic caused increases in the blood sugar values of 36, 46, 49, 51, 25 and 25 per cent (see (1), tables 7 and 8). And, again, abdominal operations have caused pronounced hyperglycemias in some of the cases recorded in table 1; thus, in experiment 28 the blood sugar value was raised to 277 mgm. per 100 cc. by laparotomy and removal of the remaining adrenal; and in experiments 32 and 33 laparotomy and dissection of the hepatic nerves, at the time of the experiment raised the blood sugar value to 263 and 229 mgm. per 100 cc., respectively (see (1), tables 1 and 2). In order for the blood sugar concentration to be thus increased in these cases it would seem to be required that the carbohydrate depots should have been well stocked.

In conclusion, the evidence presented in this and the preceding paper (1) seems to indicate a relationship of the adrenals to carbo-

hydrate mobilization, of a kind that previously mentioned. And this rela same nature as that which obtains reactions as cardiac acceleration, v inhibition, etc. These are all reactions the aid of the adrenals, although an sustains and augments them. In the possible to produce a reflex hyperglyce the coöperation of the adrenals (1); that this was accomplished by means maximal, tetanizing current applied t animal probably never experiences in it has also been shown that the less i the preparation of the animal for an binding and insertion of an artery important—produce much less augmen when there is no possibility of their tained by simultaneously stimulated normally.

Should this view of the relationship mobilization prove to be true, it will in the literature. It is no wonder tha hydrate mobilization is possible without and it is not surprising, either, that ot the same conditions. Either result m factor which seems never to have be intensity of the stimulation used to e tion.

CONCLUSI

It is shown that animals whose adr mobilized (by removing one and den and more constant blood sugar value experiments (after anesthetization, tyi cannula) than do animals that are n nervation.

This, together with the evidence pre suggests that stimuli which might be t carbohydrate mobilization, of themsel respect if they can have the sustaining lated adrenin discharge.

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kind that it has been impossible to find
nd this relationship appears to be of the
h obtains between the adrenals and such
leration, vascular constriction, intestinal
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hyperglycemia of normal intensity without
renals (1); but it must not be overlooked
l by means of an intensity of stimulation—
t applied to the sciatic nerve—such as the
eriences in actual life. On the other hand,
t the less intense stimuli which result from
imal for an experiment, and of which the
an artery cannula are probably the most
less augmentation of the blood sugar value,
y of their effects being reinforced and sus-
stimulated adrenal activity, than they do

relationship of the adrenals to carbohydrate
rue, it will clear up much of the confusion
wonder that many have found that carbo-
ible without the coöperation of the adrenals;
ner, that others have had only failure under
er result may be obtained, depending on a
to have been considered, viz., the relative
n used to effect the carbohydrate mobiliza-

CONCLUSION

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sugar values under the conditions of these
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that are normal in respect to adrenal in-

vidence presented in a preceding paper (1)
might be too weak to cause a pronounced
of themselves, may be effective in this
sustaining coöperation of a reflexly stimu-

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